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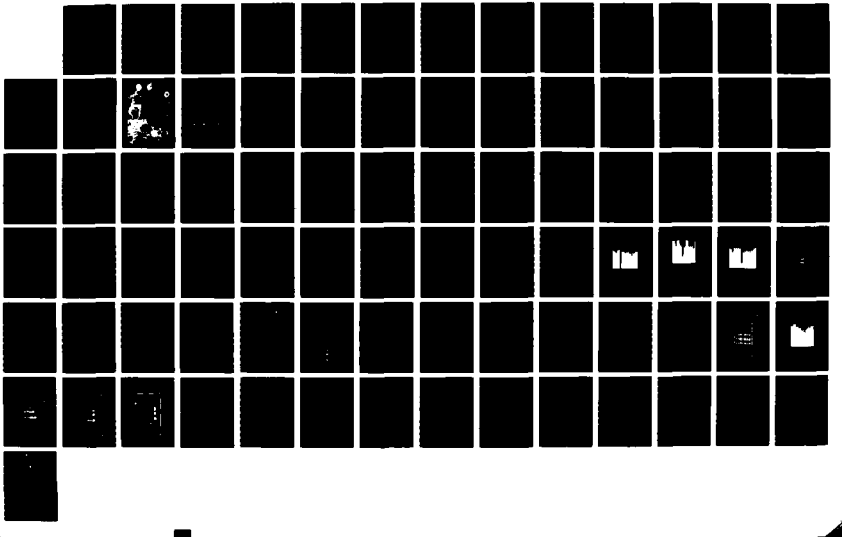

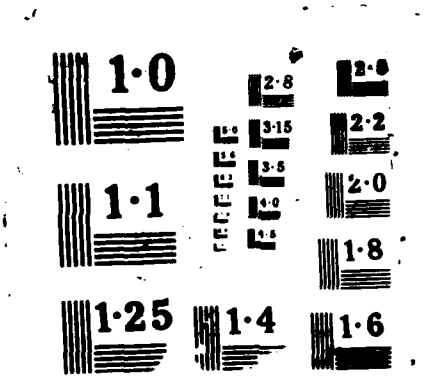
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Taxonomic characteristics and variances of a variety, of arboviruses are discussed

SUMMARY

Quaranfil is one of the few human arbovirus pathogens still of undetermined taxonomic status; it is related to Johnston Atoll virus. A virus isolated from a bird in Nigeria was added to the Quaranfil serogroup and shown to be new and different from Quaranfil and Johnston Atoll viruses by neutralization test. Morphological and physicochemical characterization of the Quaranfil serogroup viruses showed them to be spherical, 140 nm diameter RNA viruses, which bud from the plasma membrane. Virus particles had lipid envelopes which enclose ribosomelike structures. Six virus-specified polypeptides were detected by Western blot. Although they were somewhat similar to arenaviruses, the Quaranfil serogroup viruses appeared to differ from all described virus families.

The Vesiculovirus genus, family Rhabdoviridae was revised serologically. Immunofluorescence, complement-fixation, enzyme-linked immunosorbent assay and neutralization tests showed that VSV-Indiana, Cocal, Alagoas, and Maraba viruses were very closely related; that the new virus from Brazil, Carajas, was also related to these four vesiculoviruses, but less closely; and that VSV-New Jersey was quite distinct from the others. Most of these agents cause vesicular disease in cattle and some infect human beings. Carajas and Maraba viruses are of unknown pathogenicity. The demonstration of the close relationship of VSV-Indiana, Cocal, Alagoas, and Maraba viruses is important; this study demonstrated that current routine diagnostic procedures would not distinguish among the four. Possibly diagnostic tests utilizing monoclonal antibodies would be specific.

A novel approach to the taxonomy of the genus Orbivirus, family Reoviridae revealed what was predicted, that serology is an incomplete measure of the relatedness among and within the 13 serogroups and the ungrouped orbiviruses. The new approach involves RNA-RNA blot hybridization, probing with end-labeled dsRNA of all 10 (or 12 in the case of Colorado tick fever) segments. The genomic dsRNA of any isolate or any segment of the isolate may be used as a probe without production of cDNAs. Application of this technique to nearly all serogroups of orbiviruses has shown that 1) hybridization supports the current serogroup classification; serotypes which share the complement-fixation reaction are closely related in all or most of their segments, 2) serotypes which are only distantly related by complement-fixation test do not belong in a group by hybridization (e.g. Pata and Mitchell River viruses), 3) viruses from widely disparate geographic regions are often closely related in all or most of their genes by hybridization, 4) hybridization relationships of gene 2 correlated with the neutralization reaction, 5) the Kemerovo serogroup, which has been divided into complexes, represents multiple hybridization groups; these groups correspond to the complexes. This is the first comprehensive approach to the taxonomy of RNA viruses using large scale RNA-RNA hybridization.

Identification of viruses revealed a new ungrouped virus from Brazilian sand flies, a new Simbu group virus from a Brazilian coati (Carnivora), a Bunyamwera group virus from Australia — the first time a member of this group has been recognized on the Australian continent, a new Nairovirus from a Brazilian bird, a new Vesiculovirus from mosquitoes collected by members of a U.S. Army team in New Mexico, and a virus closely

related to the Australian rhabdovirus, Tibrogargan, from culicoid midges collected in Florida. It is clear that continued searching for arboviruses by scientists throughout the world continues to uncover newly recognized agents.

Three new serological techniques were developed or adapted. 1) Cultured C6/36 Aedes albopictus cells infected with dengue viruses and fixed in formalin served as excellent in situ antigens in the enzyme-linked immunosorbent assay (ELISA). 2) An arbovirus tissue culture microneutralization test was elaborated, also using ELISA as the indicator of positive and negative wells. This permitted virus neutralization testing in insect cells, and neutralization tests with viruses which did not produce plaques or cytopathic effect. 3) Adaptation of the technique of the intrasplenic route of immunization to arboviruses produced useful ELISA mouse immune response by 5 days post inoculation. This novel technique now permits reciprocal (antigen and antibody) serological testing of new arbovirus isolates in less than one week.

Hamsters were infected sequentially with three different phleboviruses -- Arumowot, Chagres, and Gabek Forest. Infection with Arumowot virus was not lethal and did not diminish the level of viremia during a subsequent infection with Chagres virus. The second infection (with Chagres virus) boosted the Arumowot antibody but did not result in detectable Gabek Forest neutralizing antibody. Gabek Forest infections were fatal, however hamsters were protected from lethal Gabek Forest infections by prior sequential infections of Arumowot and Chagres viruses. The reciprocal experiment, i.e. Chagres infection followed by Arumowot infection also resulted in viremia after each virus and in a booster effect of the Chagres antibody without inducing demonstrable Gabek Forest antibody. Four of eight of these animals were protected from challenge with Gabek Forest virus. Infection with Arumowot virus alone protected hamsters from challenge with Gabek Forest virus to a minor degree only. These results and neutralization testing of sera of these animals showed that sequential infections with non-lethal phleboviruses prolonged the time to death or protected against lethal challenge; that the neutralization test was relatively specific, even after sequential infection; and that immune enhancement was not demonstrated in vivo with phleboviruses.

The World Reference Center for Arboviruses, as in prior years, distributed viruses, antigens, antibody, cell cultures, and colonized arthropods to laboratories, both in the United States and world-wide.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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I. VIRUS CLASSIFICATION

A. FAMILY UNDETERMINED

The Quaranfil serogroup of tick-borne viruses including Ib An38918, a newly recognized member (L.T.M. Figueiredo, T. Burrage, and R.E. Shope). The Quaranfil serogroup of tick-borne arboviruses continues to be taxonomically unclassified. Quaranfil virus was isolated twice from Egyptian children with mild febrile reactions as well as from Argas ticks and birds. Johnston Atoll virus was isolated from Ornithodoros capensis ticks collected on Sand Island, Johnston Atoll in the Pacific Ocean in 1964.

Ib An 38918 virus is a newly recognized member of the Quaranfil serogroup from Nigeria. It was isolated by the staff of the Ibadan Virus Laboratory from a bird, Pleositagra vitelinus, collected at Lake Chad during 1969. It was previously identified as a member of the Quaranfil serogroup by CF test, but definitive identification as a new virus has only now been done. Its identification stimulated a study to provide new information on the group's antigenic, morphologic, physicochemical, and biological characteristics.

Antigenic characterization. Quaranfil virus was titered in Vero cells using IFA to determine positive and negative wells. The titers in log TCID50 were: day 1, 2.5; day 2, 4.5; day 3, 4.5; day 4, 5.5; days 5-8, 4.5.

Ib An38918 reacted reciprocally in the 1:10 dilution with Johnston Atoll antigen by IFA, thus confirming the earlier CF results; quantitative IFA determinations were not made. Extensive cross-reaction was also noted by ELISA as shown in Table 1. The homologous titer for each of the three Quaranfil group viruses was 2- to 8-fold higher than heterologous.

A neutralization test (NT) in cell cultures used serial 2-fold antibody dilutions and a constant amount of virus between 50 and 200 TCID50. The serum-virus mixtures were incubated 1 h 37C and added to the cell monolayers. The virus was titrated in 10-fold dilutions in the same assay. After a pre-determined incubation period the enzyme-linked immunosorbent assay (ELISA) was used to detect wells positive or negative for antigen.

The neutralization test was relatively specific (Table 2). IbAn38918 did not cross-react with either Johnston Atoll or Quaranfil viruses by this test.

In past years, attempts to show serological relationships of Quaranfil and Johnston Atoll viruses to other arboviruses by CF test were negative. The ELISA was now used to screen Quaranfil again for possible relationships. Infected CER cells constituted the antigen which was negative with 22 NIH grouping fluids: groups A, B, C, Bunyamwera, Bwamba etc., California, Anopheles A etc., phlebotomus fever, Simbu, Guama, Congo etc., Capim, Patois etc., rabies etc., Tacaribe, VSV, and polyvalent numbers 2, and 5 through 9. The antigen was positive with the polyvalent fluid containing Quaranfil antibody.

Morphology and morphogenesis. Past attempts to determine the EM ultrastructure of Quaranfil virus (F.M. Murphy, personal communication)

Table 1

Results of ELISA with Quaranfil serogroup viruses

Virus	Antibody		
	Quaranfil	Johnston Atoll	Ib An38918
Quaranfil	40,000	6,400	3,200
Johnston Atoll	1,600	12,800	1,600
Ib An38918	5,000	2,500	20,000

Table 2

Results of neutralization test with Quaranfil serogroup viruses

Virus	Antibody		
	Quaranfil	Johnston Atoll	Ib An38918
Quaranfil	1,280	40	<40
Johnston Atoll	320	320	<40
Ib An38918	<40	<40	2,560

Quaranfil virus titer 2.8 log TCID50/ml
Johnston Atoll virus titer 3.5 log TCID50/ml
Ib An38918 virus titer 2.3 log TCID50/ml

visualized particles which were of uncertain taxonomic status. Reexamination of Quaranfil virus at YARU was done by thin section electron microscopy of infected Vero cells and mouse brain. Vero cells were screened for antigen by immunofluorescence 10 days post inoculation and mouse brains were removed when the animals showed signs of disease. Antigen in the brain was confirmed by immunofluorescence using frozen sections. Tissue culture cells and 1 mm3 blocks of selected regions of infected brains were fixed with a solution containing 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at 4C. Cells were post fixed with 2% osmium tetroxide, mordanted with 0.3% tannic acid and en bloc stained with uranyl acetate. The material was dehydrated in ethanol and embedded in Epox 812, then sectioned with a diamond knife and stained with uranyl acetate and lead citrate. The sections were examined and photographed with a Philips 201 electron microscope.

For immunoelectron microscopy, the cell sheet was rinsed with PBS, fixed in the flask lightly with 1% glutaraldehyde in cacodylate buffer for 1 h at 4C, then risnsed again with PBS, incubated with 1:100 dilution of Quaranfil mouse hyperimmune ascitic fluid, and rinsed with 1% BSA-PBS. Protein A-colloidal gold conjugate was incubated with the cell sheet for 30 min, and rinsed with BSA-PBS as above for electron microscopy.

Budding profiles were observed on approximately 1 per 50 Vero cells (Figure 1). Particles were somewhat irregular and had an average diameter of 140 nm. A relatively thick surface coat was best preserved with a high initial concentration of glutaraldehyde. The surface coat was labeled specifically with Quaranfil antibody and colloidal gold (Figure 1A). The cytoplasmic surface of the budding plasma membrane was thickened in comparison to the surrounding plasma membrane. Cytoplasmic ribosomelike structures and floculent material appeared in the interstices of the budding particle (Figure 1B). Particles with the same appearance were present in the intercellular spaces of infected mouse brains (Figure 1C).

Physicochemical characterization. The sensitivity to 1:500 deoxycholate was determined for Johnston Atoll virus in Vero cell cultures using ELISA on day 6 to indicate positive and negative wells. The titer in the deoxycholate treated cultures was 2.3 log TCID50 and in the untreated controls was 3.8 log TCID50.

BUDR (a DNA inhibitor) was used at 10^{-5} M in titrations of Quaranfil, chikungunya, and vaccinia in CER cell cultures. The cultures were tested by ELISA on day 6 to determine the positive and negative wells. Quaranfil virus infectivity in BUDR was the same or higher than in controls. Vaccinia virus infectivity was reduced 10000-fold by BUDR.

Quaranfil-infected and mock infected Vero cells were washed twice with PBS and were solubilized in a buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, 2 mg/ml phenylmethylsulphonylfluoride (PMSF). The lysates were clarified by centrifugation, added to Laemmli sample buffer and boiled. The proteins were transferred to nitrocellulose using a Bio-Rad TransBlot apparatus. The blotted proteins were blocked with 3% gelatin followed by overnight incubation with 1:25 and 1:50 dilutions of the Quaranfil mouse immune ascitic fluid in 1% gelatin. The bound antibodies were detected with a horseradish peroxidase-labeled goat anti-mouse immunoglobulin using 1-chloro-4-naphtol as substrate.

Western blot analysis revealed 6 unique proteins with molecular weights of 101,500, 83,000, 59,700, 54,800, 32,000, and 18,000 present in the Quaranfil infected cells (Figure 2).

The Quaranfil serogroup consists of three viruses -- Quaranfil, Johnston Atoll, and a new member, Ib An38918 from the Lake Chad region of Nigeria. The members are distinct by neutralization test and related by CF, IFA, and ELISA. The viruses are spheres of approximately 140 nm diameter. They bud from the plasma membrane, have a lipid envelope, contain RNA, and have at least 6 virus specified proteins ranging from 18,000 to 101,500 daltons. The taxonomic status of this serogroup is uncertain, but the ribosomelike structures in the virions are reminiscent of arenaviruses.

Figure 1. Thin section electron microscopy of Quaranfil virus shows the particles budding from the plasma membrane of Vero cells (A, upper figure) labeled with Quaranfil mouse immune ascitic fluid and colloidal gold. The virions are approximately 140 nm in diameter. Cytoplasmic ribosomelike particles are present in a budding particle (B, insert). Thin section electron microscopy of Quaranfil-infected mouse brain (C, lower figure) reveals particles similar to those seen in Vero cells, but in the intercellular spaces.

Figure 2. Proteins of mock-infected and Quaranfil-infected Vero cell lysates were electrophoresed with molecular weight markers (far right columns). Western blots stained with Quaranfil immune mouse ascitic fluid (column B, 1:25; column D, 1:50) reveal proteins of 101,500, 83,000, 59,700, 54,800, 32,000 and 18,000 daltons.

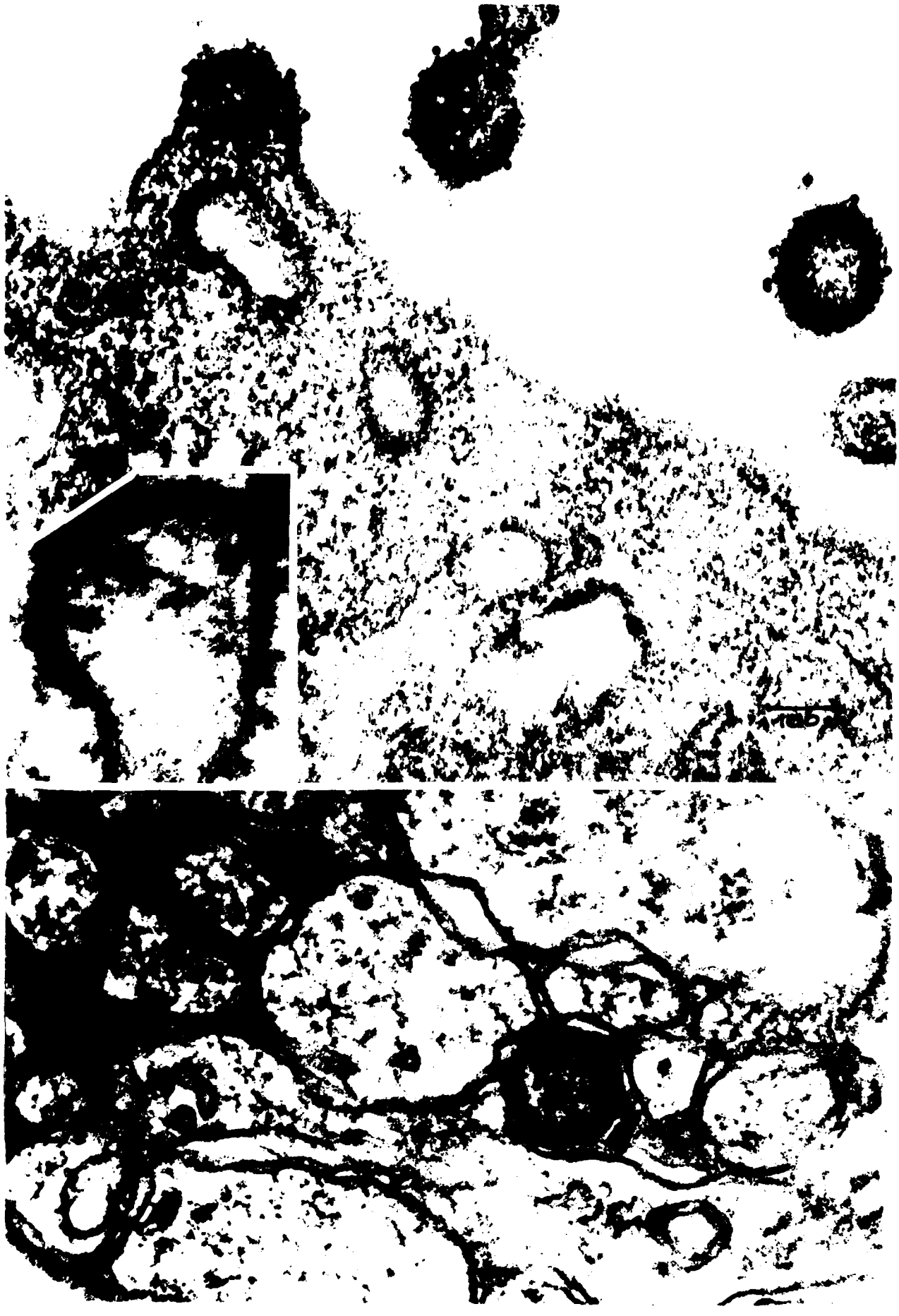
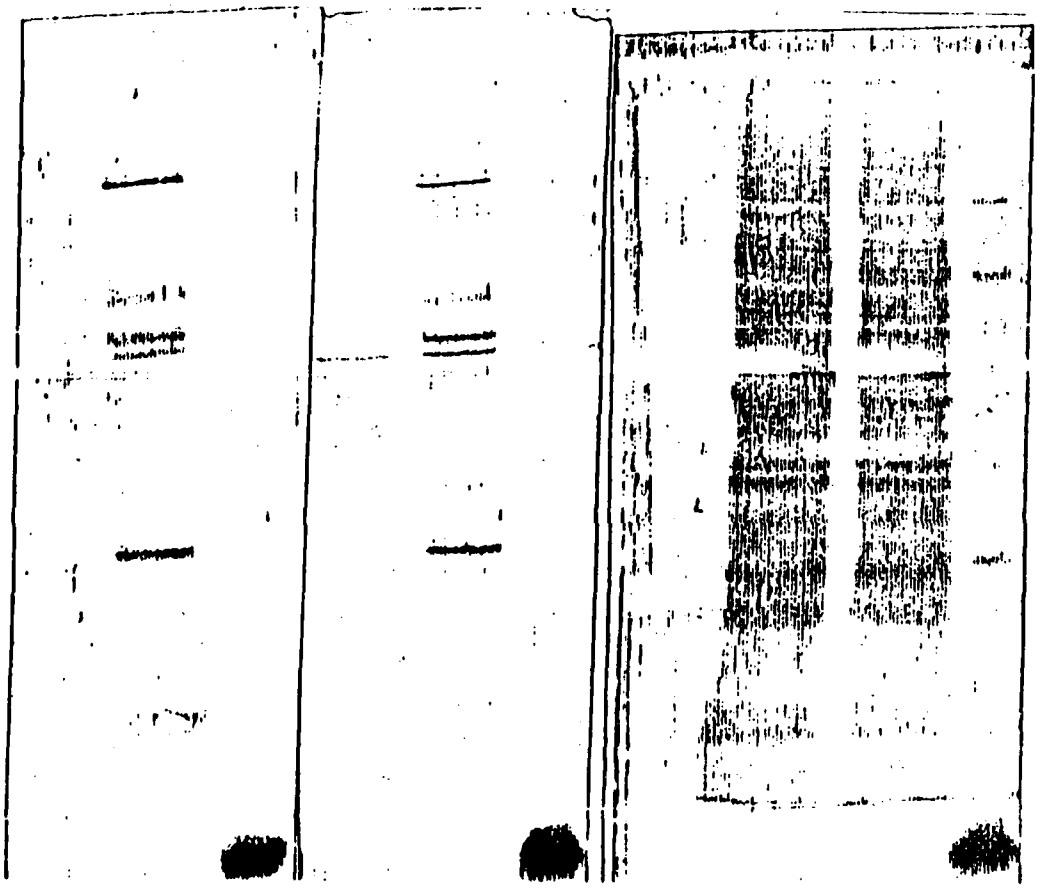


Figure 2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Vero
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Antibody 1:25
Antibody 1:50

B. RHABDOVIRIDAE, Vesiculovirus

Serological studies of the vesicular stomatitis virus (VSV) group (R.B.Tesh) Serological studies (complement-fixation, plaque reduction neutralization, indirect fluorescent antibody and enzyme-linked immunosorbent assay) were done on six related VSV group agents to determine their antigenic relationship. Results are given in Tables 3, 4, 5 and 6. These data indicate (1) that Indiana, Cocal, Alagoas and Maraba viruses are very closely related antigenically; (2) that Carajas virus is also related to the other four agents, but less closely; (3) and that New Jersey virus is quite distinct from the rest. In IFA and ELISA tests, Indiana, Cocal, Alagoas, Maraba and Carajas viruses could not be clearly differentiated. In CF and PRN tests, they could be distinguished but only if end-point titrations were done using all five immune reagents. It appears that the latter five agents comprise an Indiana complex within the VSV group (genus Vesiculovirus). New Jersey, Indiana, Cocal and Alagoas viruses have all been associated with epizootics of vesicular disease in domestic animals; the pathogenicity of Carajas and Maraba viruses for humans and/or animals is unknown.

Table 3

Results of plaque reduction neutralization tests with selected vesiculoviruses

Immune ascitic fluid

Virus	Alagoas	Indiana	Cocal	Maraba	Carajas	New Jersey
Alagoas	327,680*	320	0	160	0	0
Indiana	640	327,680	10	1,280	20	10
Cocal	160	20	5,120	320	20	0
Maraba	40	160	0	81,920	0	20
Carajas	NT	10	0	10	20,480	10
New Jersey	NT	10	0	0	20	2,621,440

*Reciprocal of highest ascitic fluid dilution producing ≥90% plaque reduction.

0 = <1:10; NT = Not tested.

Table 4

Results of complement-fixation tests with selected vesiculoviruses

	Immune ascitic fluid					
Antigen	Alagoas	Indiana	Cocal	Maraba	Carajas	New Jersey
Alagoas	≥1024*	64	16	32	0	0
Indiana	16	256	16	16	0	0
Cocal	16	8	128	8	0	0
Maraba	256	32	64	256	8	8
Carajas	0	0	0	0	64	0
New Jersey	0	0	0	0	0	≥1024

*Reciprocal of highest positive ascitic fluid dilution. All antigens were tested at a fixed dilution (1:8).

0 = <4.

Table 5

Results of indirect fluorescent antibody tests with selected vesiculoviruses

	Immune ascitic fluid					
Virus	Alagoas	Indiana	Cocal	Maraba	Carajas	New Jersey
Alagoas	≥2560*	320	160	160	40	160
Indiana	640	320	320	320	80	160
Cocal	640	320	≥2560	1280	320	320
Maraba	1280	640	1280	1280	160	160
Carajas	640	160	40	320	640	160
New Jersey	160	20	20	40	40	640

*Reciprocal of highest positive ascitic fluid dilution.

Table 6

Results of ELISA tests with selected vesiculoviruses

	Immune ascitic fluid					
Antigen	Alagoas	Indiana	Cocal	Maraba	Caragas	New Jersey
Alagoas	12,800*	1,600	400	1,600	400	20
Indiana	≥25,600	12,800	1,600	3,200	800	100
Cocal	6,400	200	≥25,600	6,400	800	50
Maraba	12,800	1,600	3,200	6,400	400	20
Carajas	200	20	50	50	3,200	<20
New Jersey	100	50	20	20	20	3,200

*Reciprocal of highest positive ascitic fluid dilution. All antigens were tested at a 1:1000 dilution.

C. REOVIRIDAE, Orbivirus

Classification and taxonomy of orbiviruses (D.L. Knudson). Table 7 represents a short listing of the current Orbivirus serogroups and isolates based upon serologic data. In concert with the serology, polyacrylamide gel electrophoresis (PAGE) and agarose gel electrophoresis of the genomic dsRNA of these viruses have proven to be useful as initial screening tools to identify presumptive orbiviruses and unidentified viruses. Recently (YARU Annual Report, 1984; 1985), RNA-RNA blot hybridization has been utilized to examine the genetic relatedness of orbiviruses within the serogroup (See Section IV).

Current classification of orbiviruses. The genus, Orbivirus, is one of several genera in the family Reoviridae (Matthews, 1982). The third edition of the International Catalogue of Arboviruses (Karabatsos, 1985) lists 61 orbiviruses which are subdivided into thirteen serogroups and one ungrouped set of isolates. While this listing is extremely useful, it is also conservative because it does not list separately multiple serotypes for all serogroups, such as, African horsesickness (AHS) and bluetongue virus (BTV). Furthermore, the listing does not represent the many isolations of the seemingly similar isolates.

Much of the characterization and classification of the genus Orbivirus has been based upon serological tests (complement-fixation and neutralization) with polyacrylamide gel electrophoresis (PAGE) analyses being used to examine the large numbers of virus isolates (Borden et al, 1971; Murphy et al, 1971; Knudson, 1981; De Oliva and Knudson, 1982; Knudson et al, 1982; 1984; Travassos da Rosa et al, 1984; Chastel et al, 1984; Tesh et al, 1986). At the Yale Arbovirus Research Unit (YARU), we recognize Orbivirus serogroups and serotypes which are listed in Table 7, and their geographic distribution is presented in Table 8. Thus, complement-fixation tests have been used to group viruses into serogroups, and neutralization tests have been used to type viruses within the serogroup. While this has been an extremely powerful taxonomic tool, it suffers the criticism of measuring the relatedness only in those genes which encode these specific antigenic determinants.

Polyacrylamide profiles of members of different serogroups. The dsRNA PAGE profiles have been generated for the viruses listed in Table 7, and the PAGE profiles have been completed for a large number of the strains of various isolates. Members of the Kemerovo serogroup exhibited similar profiles by PAGE with 2 large segments, 4 middle size segments, 3 smaller segments, and 1 small segment (2-4-3-1). Corriparta exhibited a profile that was similar to that seen for Kemerovo viruses. The CTF profile was distinct from the others (4-6-1-1). The profiles of the remaining viruses, which were Changuinola (BT-436), BTV, Warrego (Ch9935), Tilligerry (NB7080), EHD-1, Palyam (IG5287), and Wallal (Ch12048) exhibited broad similarities (3-3-4). Table 9 lists the orbivirus isolates correlated with their respective dsRNA patterns. PAGE analyses of the ungrouped mosquito isolates have resulted in patterns which are similar to existing patterns and also totally new profiles have been seen. Likewise, viruses with 9 and 12 segments have been identified. The PAGE profile of the latter group of 12 segmented dsRNA viruses does not resemble the CTF pattern. Thus, new and novel virus isolates have been identified.

Available evidence indicates that the PAGE profile is a more sensitive indicator of genetic variation than are serological assays. One reason for this is that only a small subset of the ten viral genes encodes proteins which elicit immunological response, while gel analysis allows comparison of all ten (or twelve) genome segments. However, the basis for the PAGE mobility differences is not known, and appears to be the result of secondary structure, not of true molecular weight differences (Bodkin and Knudson, 1985b). Moreover, comigration of two bands in polyacrylamide gels does not indicate sequence identity. In fact, the PAGE profile appears to give only a minimal estimate of sequence heterogeneity, because segments with identical mobilities may exhibit sequence differences by oligonucleotide fingerprint analysis (Walker et al, 1980).

Genome profiles of orbiviruses in agarose. Although the cognate genes of Palyam serogroup viruses exhibit variability in their apparent molecular weights in polyacrylamide (Knudson et al, 1984), their molecular weights in agarose were similar (Bodkin and Knudson, 1985b). Denaturation of dsRNA segments with glyoxal prior to electrophoresis in agarose did not change their relative molecular weights. Cognate genes of Changuinola serogroup viruses also exhibit variability in their apparent molecular weights in polyacrylamide (Travassos da Rosa et al, 1984). Yet, their molecular weights in agarose were also similar (Bodkin and Knudson, manuscript in preparation). The genome segments of the CTF serogroup viruses also exhibit variability in their mobilities by PAGE (McCance and Knudson, unpublished). The genome profiles of CTF serogroup viruses were identical in agarose. In short, members of a serogroup exhibit identical, or nearly identical agarose profiles.

Species in orbiviruses. Gorman et al. (1983) and Gorman (1985) have discussed the importance of defining viral species in terms of isolates which interact genetically. In order to determine which isolates are sufficiently related to exchange genetic information, either in vitro reassortment of dsRNA segments must be demonstrated, or the sequence relatedness of the dsRNA segments must be determined.

Approaches to the assessment of genetic relatedness, Reassortment. Biological reassortment of dsRNA segments is a viable mechanism for generating genetic diversity in orbiviruses. In reassortment experiments with Colorado tick fever virus both in vitro and in the tick vector (in vivo) (McCance, E. F., Ph.D thesis, Yale University; Miller, McCance and Knudson, unpublished results), reassortants were demonstrated readily. While the biologic test of reassortment is an excellent measure of genetic relatedness, it is also a labor-intensive activity when the numbers of isolates are considered. Alternative approaches, such as solution hybridization, PAGE, oligonucleotide mapping, and sequence analysis have been utilized in attempts to assess the relatedness of dsRNA viruses in genes other than those encoding antigenic determinants. Of these four techniques, only PAGE is practical for examining large numbers of viral isolates. However, the relative mobilities of dsRNA segments reveal little about the sequence relationships between isolates.

Approaches to the assessment of genetic relatedness, RNA Blot Hybridization. A RNA blot hybridization procedure has been developed at YARU to determine the genetic relatedness of dsRNA viruses, to identify serotype-specific genes among closely related viruses, to classify new

isolates, and to examine the evolutionary rates for the viral genes (Bodkin and Knudson, 1985a; 1985b; 1986). This approach represents an alternative to in vitro reassortment experiments, and it has the advantage of being able to examine large numbers of orbivirus isolates rapidly. The blot hybridization allows a labeled probe to be hybridized to multiple isolates in a single experiment. Since PAGE can be used to resolve segments of orbiviruses which are comigrant in agarose gels and provides a diagnostic dsRNA profile of the viral isolate, it is the system of choice for blot hybridization analyses of these viruses. Segments are transferred from polyacrylamide to membranes and hybridized to radiolabeled genomic RNA from a single strain. The addition of [5'-^{32}P]-pCp to the 3' ends of the dsRNA segments has been used to generate radiolabeled "probes" (England and Uhlenbeck, 1978). Segments which are functionally or genetically equivalent among different isolates have been termed cognate genes (Gaillard and Joklik, 1982). When an isolated segment is used as a probe, then the cognates in the other isolates may be identified quickly. Thus, the genomic dsRNA of any isolate or any isolated segment may be used as a probe without the production of cDNAs.

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Table 7

ORBIVIRUS SEROGROUPS

AFRICAN HORSESICKNESS SEROGROUP
AHS 1
AHS 2
AHS 3
AHS 4
AHS 5
AHS 6
AHS 7
AHS 8
AHS 9 (7/60)[a]

BLUETONGUE SEROGROUP
BTV 1 (Biggarsberg)
BTV 2 (22/59)
BTV 3 (Sample B)
BTV 4 (Vaccine-batch 603)
BTV 5 (Mossop)
BTV 6 (Strathene)
BTV 7 (Utrecht)
BTV 8 (Camp)
BTV 9 (University Farm)
BTV 10 (Portugal)
BTV 11 (Nelspoot)
BTV 12 (Byenespoort)
BTV 13 (160/59)
BTV 14 (87/59)
BTV 15 (133/60)
BTV 16 (Pakistan)
BTV 17 (63-66B)
BTV 18 (South Africa)
BTV 19 (South Africa)
BTV 20 (CSIRO 19)
BTV 21 (CSIRO 154)
BTV 22 (South Africa)
BTV 23 (India)
BTV 24 (South Africa)

CHANGUINOLA SEROGROUP
Almeirim (Be Ar 389709)
Altamira (Be Ar 264277)
Caninde (Be Ar 54342)
Changuinola (BT 436)
Gurupi (Be Ar 35646)
Irituia (Be An 28873)
Jamanxi (Be Ar 243090)
Jari (Be An 385199)
Monte Dourado (Be An 385401)
Ourem (Be Ar 41067)
Purus (Be Ar 361064)
Saraca (Be Ar 385278)
- (Co Ar 2837)

COLORADO TICK FEVER SEROGROUP
CTF (Florio)
Eyach (Eyach 38)

CORRIPARTA SEROGROUP
Acado Hybrid Complex
Acado (Eth Ar 1846-64)
Bambari Hybrid Complex
Bambari (Dak Ar B3689)
Jacareacanga (Be Ar 295042)
- (Be Ar 263191)
Corriparta Hybrid Complex
Corriparta (MRM1)
- (CSIRO 76)
- (CSIRO 109)
- (CSIRO 134)

EPIZOOTIC HEMORRHAGIC DISEASE OF DEER SEROGROUP
EHD 1 (New Jersey)
EHD 2 (Alberta)
Ibaraki (Japan 2)
- (CSIRO 157)
- (CSIRO 753)
- (CSIRO 775)
- (DPP 59)
- (Ib Ar 22619)
- (Ib Ar 33853)
- (Ib Ar 49630)
- (JKT-9133)
- (XBM/67)

EQUINE ENCEPHALOSIS SEROGROUP
EE 1
EE 2
EE 3
EE 4
EE 5
EE 6
EE 7

EUBENANGEE SEROGROUP
Eubenangee (IN 1074)
Tilligerry (NB 7080)
- (CSIRO 20)
- (CSIRO 23)
- (CSIRO 32)

KEMEROVO SEROGROUP
Chenuda Complex
Baku (LEIV 46A)
Chenuda (Ar 1170)
Huacho (Ar 883)
Kala Iris (?)
Mono Lake (Ar 861)
Sixgun City (RML 52451)
Essaouira (Brest Ar T222)
Great Island Complex
Arbroath (ARB1)
Bauline (Can Ar 14)
Cape Wrath (Scot Ar 20)
Fin isolates (Fin NorV-808)
Foula (F80-2)
Great Island (Can Ar 41)
Great Saltee (GS80-7)
Inner Farne (IF-1)
Kenai (RML 71-1629)
Mill Door/79 (M326/79)
Mill Door/81 (M35/81)
Mykines (Den Ar 12)
North Clett/81 (M34/81)
Nugget (MI-14847)
Okhotskiy (LEIV 70C)
Poovoot (RML 57493-71)
Shiants (M325)
St. Abb's (FT363)
Tindholmur (Den Ar 2)
Yaquina Head (15)
Kemerovo Complex
Kemerovo (R 10)
Lipovnik (Lip 91)
Tribec (original)
Wad Medani Complex
Seletar (SM-214)
Wad Medani (Ar 492)

PALYAM SEROGROUP
Abadina (Ib Ar 22388)
Bunyip Creek (CSIRO 58)
CSIRO Village (CSIRO 11)
D'Aguilar (B 8112)
Kasba (I G 15534)
Kindia (A.V. 5020)
Marrakai (CSIRO 82)
Nyabira (792/73)
Palyam (I G 5287)
Petevo (Dak Ar TB 2032)
Vellore (68886)

UMATILLA SEROGROUP
Llano Seco (BFN 3112)
Netivot (NT-192)
Umatilla (69-V2161)

WALLAL SEROGROUP
Mudjinbarry (NT14952)
Wallal (Ch 12048)
- (CSIRO 44)

WARREGO SEROGROUP
Warrego (Ch 9935)
- (CSIRO 12)

UNGROUPED
Chobar Gorge (701700-8)
Ieri (TRVL 8762)
Ife (Ib An 57245)
Japanaut (MK 6357)
Lebombo (SA Ar 136)
Mitchell River (MRM 10434)
Orungo (Ug Mp 359)
Paroo River (GG 668)
Pata (Dak Ar B 1327)
- (Eg An 1398-61)
- (Eth Ar 1618)
- (Eth Ar 3201)
- (Eth Ar 3554)
- (T-50616)
Ungrouped Mosquito Isolates
9 Segments of dsRNA:
[5-4]
- (JKT-6466)
- (JKT-6612)
- (JKT-6501)
- (JKT-6688)
- (JKT-6502)
- (JKT-6715)
- (JKT-6539)[b]
- (JKT-6732)[b]
- (JKT-6569)
- (JKT-7577)
- (JKT-6607)[b]
10 Segments of dsRNA:
[2-2-2-2-2]
- (JKT-6854)[b]
? (JKT-7879)[b]
[2-4-3-1]
Matsu
- (JKT-8089)
- (JKT-7822)[b]
- (JKT-9891)
[3-3-3-1]
- (JKT-6512)
- (JKT-9126)[b,c]
- (JKT-8312)[c]
[3-6-1]
- (JKT-7781)
- (JKT-9393)
- (JKT-8547)
? (JKT-10087)[b]
11 Segments of dsRNA:
[3-3-4-1]
- (M14)
12 Segments of dsRNA:
[6-6]
- (JKT-6423)
- (JKT-7041)
- (JKT-6969)
[6-5-1]
- (JKT-7075)

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[a]Virus designation represents the virus name or - for none and the strain or isolate is listed parenthetically.

[b]Isolate is a mixture with 3 bands present at the top of the gel.

[c]EHD-like dsRNA profile.

Table 8

Geographic Distribution of Orbivirus Serotypes

Serogroup	Number of Serotypes	Geographic Distribution of Serotypes: Africa	Europe	Asia	Australia	North America	Central & South America	Vector(s)
African horsesickness	9	+	+	+				*Culicoides*
Bluetongue	23	+	+	+	3	5	+	*Culicoides*
Changuinola	>12						12	*Lutzomyia* mosquitoes
Colorado Tick Fever	2		1			1		ticks
Corriparta	6	2			2		2	mosquitoes
Epizootic Hemorrhagic Disease of Deer	12	4		1	5	2		*Culicoides*
Equine Encephalosis	7	7						*Culicoides*
Eubanangee	4	1			3			mosquitoes
Kemerovo:	23	2	8	4	1	8		ticks
Chenuda	7	1	2	1		3		ticks
Great Island	11		4	1	1	5		ticks
Kemerovo	3		2	1				ticks
Wad Medani	2	1		1				ticks
Palyam	10	3		3	4			*Culicoides* mosquitoes ticks
Wallal	2				2			*Culicoides* mosquitoes
Warrego	2				2			*Culicoides* mosquitoes
Umatilla	?3			1		2		mosquitoes
Ungrouped:	?	7			2	1		mosquitoes none
Mosquito isolates -	?			28				?
Totals:	>11	>26	>9	>37	22	19	>14	

Table 9

Correlation of DsRNA Profiles with Orbivirus Serogroups

Number of dsRNA Segments	General dsRNA Profile	Serogroup	Number of Serotypes	Vector(s)
9	5-4	Ungrouped mosquito isolates	?	?
10	2-2-2-2-2	Ungrouped mosquito isolates	?	?
	2-4-3-1	Corriparta	6	mosquitoes
		Kemerovo:	23	ticks
		Chenuda	7	ticks
		Great Island	11	ticks
		Kemerovo	3	ticks
		Wad Medani	2	ticks
		Ungrouped mosquito isolates	?	?
	3-3-3-1	African horsesickness	9	*Culicoides*
		Bluetongue	23	*Culicoides*
		Epizootic Hemorrhagic Disease of Deer	12	*Culicoides*
		Eubanangee	4	mosquitoes
		Wallal	2	*Culicoides* mosquitoes
		Warrego	2	*Culicoides* mosquitoes
		Umatilla	?3	mosquitoes
		Ungrouped mosquito isolates	?	?
	3-3-4	Changuinola	>1	*Lutzomyia* mosquitoes
		Palyam	10	*Culicoides* mosquitoes ticks
		Ungrouped:		
		Orungo	?	mosquitoes
	3-6-1	Ungrouped mosquito isolates	?	?
?10	?	Equine Encephalosis	7	Culicoides
?10	?	Ungrouped:	?	mosquitoes none
12	4-6-1-1	Colorado Tick Fever	2	ticks
	6-5-1	Ungrouped mosquito isolates	?	?
	6-6	Ungrouped mosquito isolates	?	?
			>111	

II. IDENTIFICATION OF VIRUSES

TOGAVIRIDAE, Alphavirus

Identification of chikungunya virus from Central African Republic (L.T.M. Figueiredo, M.A.V. Maciel, and R.E. Shope). CAR 256 virus was referred by M.E. Faran, U.S. Army Medical Research Institute for Infectious Diseases. The virus was isolated in 1984 from Aedes opok mosquitoes near Bozo, Central African Republic. Infected C6/36 and CER cells were used on day 3 as ELISA antigen. Initial screening by ELISA with 23 grouping ascitic fluids indicated that CAR 256 was an alphavirus. Of six African alphaviruses tested by ELISA, only chikungunya antibody reacted to high titer (Table 10). A neutralization test showed that CAR 256 and chikungunya were indistinguishable (Table 11).

Table 10

CAR 256 virus: results of ELISA with selected African alphaviruses

	Antibody						
Virus	CAR256	chik-ungunya	Semliki Forest	O'nyong-nyong	Middel-burg	Ndumu	Sindbis
CAR 256	100000	>12800	800	<50	<50	100	3200
Chikungunya	200000	40000	NT	NT	NT	NT	NT

NT = not tested

Table 11

Neutralization test of CAR 256 and chikungunya viruses

	Antibody	
Virus	CAR 256	Chikungunya
CAR 256	32000	32000
Chikungunya	16000	8000

BUNYAVIRIDAE, Bunyavirus

Identification of a new Simbu group virus from Brazil (L.T.M. Figueiredo and R.E. Shope). BeAn423380 virus was referred by A.A. Travassos da Rosa of the Instituto Evandro Chagas, Belem, Brazil. The virus was isolated in 1985 from Nasua nasua, (Carnivora) collected near Belem, and was identified by complement fixation test as a Simbu group virus at the Instituto Evandro Chagas. Infected C6/36 cells were used on day 5 as ELISA antigen. The antigen reacted in a screening test with Simbu grouping antibody. The results of ELISA with the New World Simbu group viruses are shown in Table 12. BeAn423380 was most closely related to Oropouche virus but could be differentiated. By neutralization test, BeAn423380 differed from Oropouche, Utinga, Mermet, and Inini viruses (Table 13) and appears to be a new member of the Simbu serogroup.

Table 12

BeAn423380 virus: results of ELISA with selected Simbu group viruses

	Antibody							
Virus	BeAn 423380	ORO	UTI	MER	INI	SAT	ING	MAN
BeAn423380	80000	400	100	1000	1000	3200	200	200
Oropouche	51200	3200	50	1000	200	1600	NT	NT
Utinga	3200	<50	400	50	100	NT	NT	NT
Mermet	800	<50	<50	12600	3200	NT	NT	NT
Inini	800	<50	<50	3200	51200	NT	NT	NT
Sathuperi	3200	<50	NT	NT	NT	51200	NT	NT

NT = not tested

Table 13

BeAn423380 virus: results of neutralization test with selected Simbu group viruses

	Antibody				
Virus	BeAn 423380	Oropouche	Utinga	Mermet	Inini
BeAn423380	1280	<40	<40	<40	<40
Oropouche	<40	80	NT	NT	NT
Utinga	<40	<40	40	<40	<40
Mermet	<40	<40	<40	2560	1280
Inini	<40	<40	<40	320	1280

NT = not tested

Identification of a Bunyamwera serogroup virus from Australia (L.T.M. Figueiredo and R.E. Shope). CSIRO 51 virus was referred by T. St.George from CSIRO, Brisbane, Australia. The virus was isolated in 1975 from Anopheles bancroftii mosquitoes collected at Beatrice Hill, Northern Territory, Australia. Rabbit serum to CSIRO 51 was also supplied by T. St. George. Infected C6/36 and Vero cells were used on day 5 as ELISA antigens. Screening with grouping ascitic fluids indicated that CSIRO 51 belonged to the Bunyamwera serogroup. ELISA with Bunyamwera, Batai, and Germiston viruses showed that CSIRO 51 was related, but quite distinct from these agents (Table 14). Neutralization tests (Table 15) failed to show cross-reactions although the CSIRO 51 serum did not neutralize itself. Further tests are needed for definitive identification of CSIRO 51 virus. This is the first record of a Bunyamwera serogroup virus from Australia.

Table 14

CSIRO 51 virus: ELISA with selected Bunyamwera group viruses

	Antibody			
Virus	CSIRO 51	Bunyamwera	Germiston	Batai
CSIRO 51	>6400	200	100	400
Bunyamwera	3200	160000	200	800
Germiston	400	800	1600	100
Batai	400	12800	2000	800

Table 15

CSIRO 51 virus: results of neutralization tests with selected Bunyamwera group viruses

	Antibody			
Virus	CSIRO 51	Bunyamwera	Germiston	Batai
CSIRO 51	<100	<40	<40	<40
Bunyamwera	<100	10240	<40	40
Germiston	<100	<50	100	<50
Batai	<100	<50	<50	3200

BUNYAVIRIDAE, Nairovirus

Identification of a nairovirus from Brazil (L.T.M. Figueiredo and R.E. Shope). Belem virus, BeAn141106, was referred by A.A. Travassos da Rosa of the Instituto Evandro Chagas, Belem, Brazil. The virus was isolated in 1968 from plasma of Pyriglena leucoptera, a bird, near Belem. It is registered as an ungrouped agent in the International Catalogue of Arboviruses. Infected Vero and CER cells were used on day 6 as ELISA antigens. The antigen reacted with the NIH polyvalent Congo-Hazara-Dugbe-Bhanja ascitic fluid and the Polyvalent 2 ascitic fluid (which contains Belem antibody). ELISA with Congo, Avalon, Clo Mor, Soldado, Dugbe, Kao Shuan, and Bandia revealed cross-reaction with Congo, Avalon and Clo Mor antibodies (Table 16). Neutralization tests with Congo, Avalon, and Clo Mor viruses failed to show relationships, but the homologous antibody (Table 17) titers were only 1:20 for these viruses. It thus appears that Belem virus is a nairovirus, but its precise placement in the genus is not yet determined.

Table 16

Belem virus: ELISA results with selected nairoviruses

	Antibody							
Virus	Belem	Congo	Avalon	Clo Mor	Soldado	Dugbe	K.Shuan	Bandia
Belem	320	200	800	50	<50	<50	<50	<50
Congo	<40	640	NT	NT	NT	NT	NT	NT
Avalon	<50	NT	1600	<50	NT	NT	NT	NT
Clo Mor	50	50	NT	800	NT	NT	NT	NT

NT = not tested

Table 17

Belem virus: results of neutralization tests of selected nairoviruses

	Antibody			
Virus	Belem	Congo	Avalon	Clo Mor
Belem	320	<20	<20	<20
Congo	<20	20	NT	NT
Avalon	<20	NT	20	NT
Clo Mor	<20	NT	NT	20

NT = not tested

BUNYAVIRIDAE, Phlebovirus

SA Ar 13532 virus from South Africa (R.B.Tesh) A virus designated SA Ar 13532, isolated from mosquitoes in South Africa, was submitted by Dr. B.M. McIntosh, National Institute of Virology, Sandringham. SA Ar 13532 had tentatively been identified in South Africa as Arumowot-like. Accordingly, it was tested by plaque reduction neutralization test (PRNT) against the prototype Arumowot strain, Ar 1284-64.

Results are shown below:

Virus	SA Ar 13532	Arumowot
SA Ar 13532	1:2560*	1:160
Arumowot	1:1280	1:320

*Highest MIAF dilution producing 90% plaque reduction.

Results of the PRNT indicate that SA Ar 13532 is indistinguishable from the prototype Arumowot virus strain.

Identification of three phlebotomus fever group virus isolates from Cyprus (R.B.Tesh) Three phlebotomus fever serogroup viruses (R-3, R-18 and RM-09) were submitted for identification by Dr. B. Niklasson, The National Bacteriological Laboratory, Stockholm, Sweden. Virus strains R-3 and R-18 were isolated from the blood of sick Swedish soldiers in the United Nations Peace Keeping Force on Cyprus. Strain RM-09 was isolated from a pool of phlebotomine sand flies collected on the same island. Results of PRN and IFA tests done on the three viruses indicated the following: isolates R-18 and RM-09 are strains of sandfly fever-Sicilian virus, and R-3 is a member of the sandfly fever-Naples complex.

RHABDOVIRIDAE, Vesiculovirus

Tentative identification of a new Vesiculovirus from the United States (R.B.Tesh and C.H.Calisher) Virus strain 85-488 NM was isolated from mosquitoes collected in the state of New Mexico by Dr. G. Clark, U.S.Army Medical Research Institute of Infectious Diseases. In preliminary studies by electron microscopy, 85-488 NM was shown to have rhabdovirus morphology.

Initially, 85-488 NM viral antigen (infected Vero cells) was screened by IFAT against a variety of specific rhabdovirus hyperimmune mouse ascitic fluids. Positive reactions were obtained only with the vesiculovirus immune reagents. The positive ascitic fuids and their relative intensity of fluorescence with 85-488 NM antigen are noted below.

Ascitic fluid (1:10 screening dilution)	Intensity of fluorescence with 85-488 NM antigen
Isfahan	+ (weak)
Jug Bogdanovac	1+
VSV-Indiana	+ (weak)
Jurona	2+
Piry	+ (weak)
Perinet	1+
Chandipura	2+
VSV-New Jersey	1+
Carajas	1+
Maraba	1+
VSV-Alagoas	1+
Cocal	+ (weak)
85-488 NM	4+

85-488 NM hyperimmune mouse ascitic fluid was then tested by IFAT against the following vesiculovirus antigens: VSV-Indiana, VSV-New Jersey, Perinet, Jug Bogdanovac, Piry and Isfahan. It was **positive** with all of them.

The following mouse immune ascitic fluids were then tested for neutralizing activity against 85-488 NM virus by plaque reduction neutralization test: VSV-Alagoas, VSV-New Jersey, Isfahan, Piry, Chandipura, Jurona, Jug Bogdanovac, Carajas, Maraba, Cocal, Perinet, AG83-1342, and Porton-S. All ascitic fluids were negative at a 1:10 screening dilution.

It appears that 85-488 NM, designated Malpais Spring, is a new member of the VSV serogroup (genus Vesiculovirus). In newborn mice and Vero vells it produces rapid death/CPE, like most of the other VSV group viruses. CF tests with other vesiculoviruses are planned in the near future.

Identification of a Tibrogargan-like virus from the United States (R.B.Tesh and C.H.Calisher) A virus, designated BT82-55, was isolated from culicoid midges in Florida by Dr. P. Gibbs, University of Florida Veterinary School, Gainesville. By electron microscopy at CDC it was shown to be a rhabdovirus.

Initially, BT82-55 viral antigen (infected Vero cells) was tested by IFAT against the following mouse hyperimmune ascitic fluids: Almpiwar, Mosqueiro, Kimberley, Marco, Parry Creek, Tibrogargan, Paroo River, Chandipura, Keuraliba, VSV-New Jersey, Cuiaba, Porton-S, Perinet, Charleville, Inhangapi, Kamese, Mokola, Piry, Jurona, Klamath, Isfahan, Yata, Jug Bogdanovac, La Joya, Hart Park, VSV-Indiana, bovine ephemeral fever, Gray Lodge, Navarro, Aruac, 85-488NM and BT82-55. Ascitic fluids were screened at a 1:10 dilution. All were negative except Tibrogargan and the homologous MIAF.

Tibrogargan and BT82-55 mouse ascitic fluids were then titrated against both antigens with the following results.

	MIAF	
Viral Antigen	Tibrogargan	BT82-55
Tibrogargan	1:1280	1:1280
BT82-55	1:1280	1:1280

By IFAT, Tibrogargan and BT82-55 could not be differentiated; however, biologically they behave quite differently. Their pathogenicity for newborn mice, plaque morphology and growth in Vero cells are quite distinct, so they may be different. CF tests should be helpful in differentiating them. BT82-55 does not produce readable plaques in Vero cells, so PRNT is not be possible.

FAMILY REOVIRIDAE, Orbivirus

Identification of two Chenuda complex viruses from France. (A.J. Main). Brest ArT222 and Brest ArT598, isolated from ticks, were referred for identification by Dr. Claude Chastel of the Faculte de Medecine de Brest. Complement fixation tests indicated that they were in the Chenuda complex. Plaque reduction neutralization tests with 90% endpoints produced the following results:

	Ascitic Fluids			
Viruses	T222	T598	Chenuda	SixGun City
Brest ArT222	160	<10	<10	<10
Brest ArT598	<10	160	20	<10
Chenuda	20	10	320	not done
SixGun City	<10	<10	not done	80

Ascitic fluids of Mono Lake and Huacho did not neutralize T222 and T598 viruses. Baku ascitic fluid titered <10 with T222 and 40 with T598. The Mono Lake, Huacho, and Baku homologous titers have not yet been determined, but assuming these ascitic fluids are potent, the French viruses appear to be two new members of the Chenuda complex.

FAMILY UNDETERMINED

Identification of a new arbovirus from Brazil (R.B.Tesh and W.R.Chen). BeAr 421710 virus, isolated from phlebotomine sand flies in Brazil, was submitted by A. Travassos da Rosa, Instituto Evandro Chagas, Belem, for study. BeAr 421710 antigen was examined by complement-fixation (CF) and/or indirect fluorescent antibody (IFA) tests aginst the following mouse immune ascitic fluids (MIAF): Serogroup A,B and C grouping reagents; NIH polyvalent grouping sera #2,5,6,7,8 and 9; phlebotomus fever, Palyam, Tacaribe, Simbu, Bunyamwera, Capim, Kemerovo, vesicular stomatitis, California, Patois, Anopheles A, and Changuinola group reagents; polyvalent rabies, LCM and herpes; and Charleville, BeH 151, Mapuera, Mojui dos Campos, Inhangapi, Para, Bwamba, Enseada, Chagres, Rio Grande, Sripur, Bradypus 4, VSV-Indiana, VSV-New Jersey and BeAr 421710 specific MIAF. Positive reactions were obtained in both CF and IFA tests with the homologous MIAF. A weakly positive reaction was obtained in IFA with Charleville MIAF and BeAr 421710 antigen. All other results were negative. The data indicate that BeAr 421710 is a probable new arbovirus, possibly distantly related to Charleville virus.

III. CHARACTERIZATION OF MONOCLONAL ANTIBODIES

Reactivity of monoclonal antibodies prepared against sandfly fever-Sicilian virus (R.B.Tesh) A battery of 16 mouse monoclonal antibodies, prepared to the prototype strain of sandfly fever-Sicilian virus, was sent by Dr. J. Meegan, U.S.Army Medical Research Institute of Infectious Diseases, Frederick, Maryland, for testing. These monoclonal reagents were tested by IFA against Vero cells (antigen) infected with five different phlebovirus strains (Sicilian prototype, Sicilian strain 91026-E, Sicilian strain R-18, Sicilian strain I-701735 and Corfu virus). The origins of these virus strains are shown in Table 18, as are the results.

Table 18

Reactivity by IFAT of 16 Sicilian sandfly fever virus monoclonals with five virus strains in the Sicilian complex (Bunyaviridae: Phlebovirus)

		ANTIGEN				
Monoclonal AB	(dilution)	Sicilian	91026-E	R-18	I-701735	Corfu
SF2-3G2-1-1-1	(1:100)	++	++	+	+	+
SF2-7G6-2-1-2A	(1:100)	+	+	+	+	+
SF2-5D3-1-2-1	(1:10)	++	++	++	++	0
SF2-9B4-1-2-2A	(1:1000)	+	+	+	+	0
SF2-3D7-1-1-1	(1:100)	+	++	+	++	0
SF2-5B8-2-1	(1:100)	++	+	+	++	±
SF2-2B6-1-1-1	(1:100)	+	+	+	++	0
SF2-3B5-2-1-2	(1:100)	++	++	+	++	0
SF2-10C6-2-2-A	(1:100)	++	++	++	++	0
SF2-2B6-3-2-1	(1:100)	+	+	+	+	0
SF2-3E2-2-1-1	(1:10)	+	+	±	+	0
SF2-10C11-1-2-1A	(1:100)	+	+	±	+	±
SF2-9C3-1-1-2A	(1:100)	++	±	0	+	±
SF2-3D7-1-1-3	(1:100)	++	±	++	++	0
SF2-4B11-3-2-3	(1:100)	+	0	0	0	0
SF2-3E2-7-1-1	(1:100)	+	+	+	++	0

Virus strain identification

Sicilian - Prototype strain recovered from human serum in Sicily in 1943.
91026-E - Recovered from pool of Phlebotomus papatasi collected in Iran in 1975.
R-18 - Recovered from sandfly fever patient in Cyprus in 1985.
I-701735 - Recovered from human serum in India in 1970.
Corfu - Recovered from sandfly pool collected in Corfu Island, Greece in 1981.

IV. DEVELOPMENT OF NEW TECHNIQUES

An enzyme immunoassay for dengue antibody using infected cultured mosquito cells as antigen (L.T.M. Figueiredo and R.E. Shope). A simple technique was developed to detect dengue antibody using dengue virus-infected cultured cells as antigen. The technique is an adaptation of EIAs proposed recently for antigen detection of other viruses in cell cultures. The use of infected cultured cells for EIA antigen avoids a virus purification step or the need for an antigen capture step.

Dengue-1 virus strain 1413 was obtained from D.J. Gubler. It was isolated from human serum in Haiti in 1983 and was passed twice in mosquitoes and once in C6/36 Aedes albopictus cells. Dengue-2 virus strain 1232 was obtained from L. Rosen. It was isolated from human serum in Indonesia in 1978 and was passed twice in mosquitoes and once in C6/36 cells.

Dengue-1 and dengue-2 hyperimmune mouse immune ascitic fluids were prepared using prototype mouse-adapted viruses.

The following human sera were used: A from a person 10 years post dengue infection plus vaccinations for yellow fever and Japanese encephalitis; B from a person two or more years after dengue-2 infection; C from a yellow fever convalescent patient; D, E, and F from persons after inoculation with 17D yellow fever vaccine; G, H, and I were pooled sera obtained in 1982 from 40 residents of Cuba following epidemics of dengue-1 and dengue-2; and J from a North American resident with no known flavivirus exposure.

CER, LLC-MK2, Vero, and BHK-21 cells were used in developmental trials in 96-well microplates at a density of 10^5 cells per well. C6/36 cells were plated at a density of 2×10^4 cells per well. After 24 h the cells were infected either with log10 dilutions of dengue viruses or with 100 TCID50 of virus. Alternate columns of the microplate wells contained cells which were uninfected. After suitable incubation periods, (usually 6 days) the microplate wells each containing 200 µl, received 100 µl of neutral buffered formalin pH 7 (37-40% formaldehyde 100 ml, sodium phosphate dibasic-anhydrous 6.5 g, sodium phosphate monobasic 4.0 g, and distilled water 900 ml) and were held overnight at 4C. Within 18-24 h of fixation, the cells were washed twice with phosphate buffered saline (PBS). Some microplates were processed immediately for EIA. Others were air dried, put in plastic bags and sealed in a nitrogen atmosphere. The microplates containing infected and non-infected fixed cells were stored at 25C, 4C, -20C, or -70C.

For EIA the cells were saturated with 200 µl of blocking buffers (.5% bovine serum albumin [BSA] in PBS; .05% tween 20 in PBS; 5% horse serum in tween-PBS; or 3% gelatin in PBS). A comparison of the 4 blocking buffers used in combination with dengue-1 virus-infected and uninfected C6/36 cells showed that none of the blockers was significantly better than any other. After 1 h at 37C, antiserum diluted in PBS containing .5% BSA was added for 1 h. Horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-human immunoglobulin (diluted 1:2000) in .5% BSA-PBS was added for 1 h at 37C. Results were read visually and spectrophotometrically (414 nm) 20 to 30 min after the addition of ABTS substrate to wells that had been washed 5 times with .05% tween 20 in PBS.

Optical density (OD) values were considered significant if they exceeded by 3 S.D. the mean value for control wells in the same assay. Virus titers were calculated by the method of Reed and Muench.

Some sera reacted non-specifically with C6/36 cells; for these, one hundred µl of human sera were diluted 1:10 in .5% BSA-PBS and adsorbed at 4C overnight with previously pelleted, washed C6/36 cells.

To test for residual virus after formalin treatment, C6/36 cells growing in 25 cm2 flasks were infected with 1000 TCID50 of dengue-1 or dengue-2 virus. Formalin was added to the flasks 6 days after virus inoculation (final concentration 3.3%). A similar volume of PBS was added to control flasks. After 18 h at 4C, the cells were washed, disrupted by one cycle of freezing and thawing and centrifuged at 10,000 xg for 10 min. Fifty µl of supernatants and log10 dilutions were used to infect C6/36 cell cultures growing in microplates. The cells were processed 6 days later by EIA.

To determine which cell line was most permissive to dengue virus, CER, BHK-21, Vero, C6/36, and LLC-MK2 cells in microplates were exposed to log10 dilutions of dengue-2 virus. EIAs were performed 6 days post-infection. Dengue-2 virus titers were significantly higher in C6/36 cells than in the other cell lines (C6/36, 5.7 log10/ml; BHK-21, 3.5 log10/ml; Vero, 2.8 log10/ml). CER and LLC-MK2 cells yielded no reaction product. All cell monolayers adhered to the plastic wells. Based on these data, C6/36 cells were chosen for routine use in the EIA.

Development of dengue virus antigen in C6/36 cells was tested in order to determine the optimal incubation period. Serial dilutions of dengue-1 and 2 viruses were added to one day-old C6/36 cells and the cells were fixed daily for processing by EIA. For dengue-1 virus, the highest titers were obtained at 6-10 days post-infection (Table 19). The peak dengue-2 virus titer ($10^{5.7}$ log10 per ml) was found at 6 days post-inoculation. A 6-day incubation was chosen for future EIA's.

OD values for human sera with uninfected C6/36 cells were 35-48% higher than comparable OD values with dengue-1 MIAF at a 1:100 dilution. Human sera diluted 1:100 and tested on uninfected C6/36 cells yielded ODs greater than 0.60. Nonspecific binding of human sera to C6/36 cells, possibly because of sensitization by mosquito bites, was thought probable cause for the high background. To attempt to reduce background, human sera were absorbed with C6/36 cells. Human sera diluted 1:10 were adsorbed at 4C overnight, with different numbers of C6/36 cells. Adsorption with C6/36 cells reduced the OD values with uninfected cells as shown in Table 20. The effect seemed optimal at $5x10^6$ cells (46% reduction for serum A).

Ten adsorbed human sera with reduced background were tested with dengue-1 and dengue-2 infected C6/36 cells. Sera G (1:1000 titer), H 1:1000 titer), and I (1:1600 or greater titer) were positive for dengue-1 antibodies and sera A (1:100 titer), B (1:12,800 titer), C (1:1600 titer), and E (1:400 titer) were positive for dengue-2 antibodies (Table 21).

To determine whether dengue antigen in the form of infected C6/36 cells was stable, plates were stored at different temperatures after formalin fixation. An EIA was performed with cells immediately after

fixation and with cells after 2 months' storage at varying temperatures. The dengue-1 MIAF titer on dengue virus-infected C6/36 cells was 1:6400 on fresh cells. The MIAF titer decreased greater than 32-fold when tested on cells stored for two months at room temperature (titer 1:200) and 4C (titer <1:100). The MIAF titer did not change significantly in assays of cells held for two months at -20C and -70C (titers 1:12800).

Infectivity was not detected in cell lysates derived from formalin-treated cells. Lysates of PBS-treated cells yielded $10^{4.9}$ TCID50/ml (dengue-1 virus) and $10^{5.8}$ TCID50/ml (dengue-2 virus).

The EIA described here using infected cultured cells for dengue antibody detection has many advantages over conventional antibody capture EIAs and other tests: it eliminates solid phase coating with dengue virus and laborious antigen preparation; it permits screening of large numbers of sera faster and more easily than by HI or PRNT; 3.3% formalin inactivates viral infectivity, thereby reducing the potential hazard of laboratory infection; C6/36 cells infected in microplates with dengue virus and fixed with formalin can be stored for at least 2 months at minus 20C or minus 70C and also can be transported for use in remote dengue virus endemic or epidemic areas.

The finding of higher dengue-1 virus titers in C6/36 cells than in other cell lines is consistent with results of other studies of dengue virus infection of this cell line. C6/36 cells have advantages over other cell lines: they grow rapidly, can be maintained in the tropics without an incubator, and contain a high percentage of antigen-positive cells in spite of no evident cytopathic effect.

Elevated OD values for wells containing uninfected C6/36 cells and treated with human sera were a problem in the dengue virus EIA. The background reduction associated with adsorption of human sera with C6/36 cells was satisfactory although the adsorption procedure added another step to the assay.

The EIA detected dengue-1 antibodies present in sera from the 3 pools of Cuban dengue virus-infected workers. The EIA also detected dengue-2 antibodies in sera from two dengue-2 virus-infected patients, one yellow fever convalescent patient and one of two yellow fever vaccinees. Although these experiments were not designed to test the specificity of the EIA, it is clear that the test is cross-reactive among flavivirus antibodies. Dengue-1 antibodies were not detected in sera from a person without antecedent flavivirus infection, or from one of two yellow fever vaccinees. The detection of dengue-2 antibody at a high dilution (1:12800) of human serum B suggests that the EIA is a very sensitive test. The test is applicable as a rapid screening procedure for flavivirus antibody in epidemiologic studies.

Table 19

Development of dengue 1 virus in C6/36 cells as determined by EIA with dengue 1 mouse immune ascitic fluid

Days post inoculation	Virus titers (log10/ml)
1	1.8
2	<2.3
3	3.6
4	4.7
5	5.2
6	5.9
7	6.0
8	6.3
9	5.9
10	5.9

Table 20

OD values of human sera (1:100 dilution) non-absorbed and absorbed with different numbers of C6/36 cells, and processed by EIA

Absorption	OD of serum				
	A	D	E	F	J
none	.73	.67	.90	.65	.70
1x10 cells	.52	.53	.75	.54	.41
2x10 cells	.48	.51	.68	.53	.46
5x10 cells	.39	.52	.55	.45	.39
1x10 cells	.39	.52	.44	.35	.43

Table 21

ELISA reactions of human sera to dengue antigens

Serum	Antigens		Flavivirus exposure history
	Dengue 1	Dengue 2	
A	<100	100	Dengue infection 10 years before, plus Japanese encephalitis vaccine and multiple yellow fever vaccinations
B	not tested	12,800	Dengue-2 infection >2 years before
C	<100	1,600	Yellow fever convalescent serum
D	not tested	<50	Yellow fever vaccination
E	200	400	Multiple yellow fever vaccinations
F	<100	<100	Yellow fever vaccination
G	1,000	100	Pooled sera from Cuba post dengue 1 and dengue 2 epidemics
H	1,000	100	Pooled sera from Cuba post dengue 1 and dengue 2 epidemics
I	>1,600	100	Pooled sera from Cuba post dengue 1 and dengue 2 epidemics
J	<100	<100	No known exposure

An arbovirus tissue culture neutralization test using ELISA as indicator (L.T.M. Figueiredo, M.A.V. Maciel, and R.E. Shope). The microneutralization test has been modified in order to study new isolates which do not form plaques under agar, or which replicate in mosquito cells, but not in vertebrate cells. The ELISA has been adapted as a method of detecting antigen in virus-infected cell cultures, and used as an indicator for the test.

The test was used to identify 4 viruses described earlier in the report. The viruses, referred for identification to the Yale Arbovirus Research Unit, served as models for development of the technique. Be An 423380 virus, was isolated from Nasua nasua edentate in 1985; and Belem virus was isolated from Pyriglena leucoptera bird in 1968. Both were isolated near Belem and referred by A. Travassos da Rosa, Instituto Evandro Chagas, Belem, Brazil. CAR 256 virus was isolated from Aedes opok mosquitoes in Bozo, Central African Republic in 1984. It was referred by M.E. Faran of the U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Maryland. CSIRO 51 virus was isolated from Anopheles bancroftii mosquitoes from Beatrice Hill, NT, Australia in 1975 and referred by T. St.George of CSIRO, Australia.

Virus from newborn mouse brain tissue was inoculated into Vero, CER, and Aedes albopictus C6/36 cell cultures. After two passages, the cells were scraped from the flasks into the media and disrupted by freezing and thawing. The supernatant fluids were clarified by centrifugation for 10 minutes 10000 x g, aliquoted, and stored at -70C as virus stocks.

Mice were immunized with infected newborn mouse brain tissue by 4 i.p. inoculations with Freund's complete adjuvant at weekly intervals. Ascitic fluids induced by an additional inoculation of adjuvant, as well as sera, were collected approximately one month after initiation of immunization.

Vero and CER cells were grown in minimal essential medium containing equal parts of Hanks' and Earle's salts (Grand Island Biological Company, [GIBCO] Grand Island, NY), 1% L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 g/ml of streptomycin. Vero cells were maintained at 37C in 5% CO2 humidified atmosphere.

Neutralization tests were done in Vero or CER cells (see above) or in Aedes albopictus C6/36 mosquito cells which were grown in Leibowitz L15 medium (Grand Island Biological Co. [Gibco], Grand Island, NY) containing 10% heat-inactivated fetal bovine serum, 10% tryptose phosphate broth, 100 U/ml of penicillin and 100 mg/ml of streptomycin. The C6/36 cells were maintained at 28C in a humidified atmosphere. Viruses from newborn mouse brain were inoculated into the C6/36 cells and incubated for 5 days. The infected cells were then scraped into the medium and disrupted by freezing and thawing, centrifuged for 10 minutes at 10000 x g, and the supernatant fluid was aliquoted and stored at -70C as virus stock.

The neutralization tests were carried out in C6/36 cells in 96-well cluster dishes (Corning 25860, Corning, N.Y.). Cells were seeded at a density of 2×10^4 cells per well and 24 hours later the cells were infected. Antibody was mixed with 100 TCID50 of virus and the mixtures were incubated 1 hour at 37C, then added to the microplates. A titration of virus was done simultaneously. The plates were incubated 5 days and then processed by ELISA. Titers were calculated by the method of Reed and Muench.

ELISA to detect infected and uninfected wells of the neutralization test was performed by fixing the C6/36 cells to the 96-well cluster dishes. Virus-infected and control mock-infected cells were fixed at 4C for 18-24 hours in 3.3% neutral buffered formalin (37-40% formaldehyde, 100 ml; sodium phosphate dibasic-anhydrous, 6.5 g; sodium phosphate monobasic, 4.0 g; distilled water, 900 ml).

The ELISA was performed with 1 hour incubations, 100 ul volumes, diluent consisting of phosphate buffered saline with 0.5% bovine serum albumin, and 3-5 washes per step. To the formalin-fixed virus- and mock-infected antigens in the cluster dishes were added stepwise a) homologous mouse hyperimmune ascitic fluid, b) horseradish peroxidase-conjugated anti-mouse IgG (Tago Inc., Burlingame, CA; diluted 1:2000), and c) ABTS substrate. OD values were determined after 20 minutes, at 414 nm on a Multiscan Titertech ELISA reader, and were considered positive if they exceeded by 3 SD the mean values for mock-infected control wells in the same assay.

The four unknown viruses were initially grouped using NIH grouping fluids; BeAn423380 reacted with the group Simbu fluid; CAR 256, group A; CSIRO 51, group Bunyamwera; and Belem virus reacted with the Congo polyvalent ascitic fluid. The viruses were then tested by the above neutralization test with members of the serogroups represented in the grouping fluids. The results are shown in Tables 11, 13, 15, and 17 .

Intrasplenic immunization of mice with a subtype of Cache Valley virus (L.T.M. Figuieredo, R.E. Shope, and G.H. Tignor). With the advent of rapid diagnostic techniques such as IFA and ELISA, the bottleneck in determining reciprocal cross-reactions of arboviruses is the time required to prepare immune reagents. Rapid techniques for immunization of mice have been adapted from a method used in preparation of monoclonal antibodies (Spitz, M., Spitz, L, Thorpe, R. and Eugui, E., 1984. Intrasplenic primary immunization for the production of monoclonal antibodies, J. Immunol. Methods 70:39-43).

A subtype of Cache Valley, MSP-18 virus, was referred for study by J.A. Mangiafico, USAMRIID, Fort Detrick, MD and was used for the experiments. Six 7-week old CF1 outbred Swiss mice were immunized by the intrasplenic route with MSP-18 virus. They were anesthetized with methoxyflurane, the abdomen was opened by a 1.5 cm incision, and the spleen was exposed. A suspension in PBS of 0.1 ml of 10% infected suckling mouse brain was inoculated through a 27 guage needle directly into the spleen. The abdominal wall was closed with small surgical clips.

Three additional mice were immunized ip with aliquots of the same inoculum for comparative study.

Three animals from the intrasplenic group and two from the intraperitoneal group were boosted on day 5 with 0.1 ml of 10% infected mouse brain suspension in PBS. Two mice from the intrasplenic group and one mouse from the intraperitoneal group received a booster inoculation ip on day 10. Animals were bled from the heart 5 and 10 days after the initial immunization, and ascitic fluid was induced with sarcoma-180/TG on day 15 and collected by paracentesis on day 20 of the immunization schedule.

The sera and ascitic fluids were tested by neutralization test in C6/36 cells, using ELISA to detect positive and negative wells. Antibodies were also tested by ELISA; C6/36 cells fixed in 3.3% formalin served as ELISA antigen. OD values were determined on a titertech ELISA reader and were considered positive if they exceeded by 3 SD the mean value for control wells in the same assay.

Neutralization tests were done on the sera of mice numbers 1 and 8 (Table 22). On day 5, serum of mouse 1 which had ELISA titer of 10000 and of mouse 8 which had ELISA titer of 400 did not neutralize MSP-18 virus. By day 21 the ascitic fluids had neutralizing titers respectively of 640 and 160.

Table 23 shows the ELISA results. On day five, intrasplenic inoculated mice had IgG titers ranging from 400-2000 and IgM titers from 100-800. Intraperitoneal-inoculated mice had lower titers. By day 10, intrasplenic-inoculated animals boosted ip on day 5 had IgG titers ranging from 1600-10000, and those not boosted titered 800-1600. IgM titers fell by day 10 in both groups.

Ascitic fluid IgG titers on day 21 in boosted animals were 3200, and in a single animal which was not boosted was 100. A mouse immunized ip and boosted had IgG titer of 2000, not significantly different from the corresponding intrasplenic animals by day 21.

The relatively high ELISA titers by day 5 or earlier of sera of mice immunized intrasplenically are useful for the early and rapid definitive identification of arboviruses. The degree of cross-reactivity of these early antibodies remains to be determined, but experience with early sera from conventional immunization indicates that they may be serotype specific.

Table 22

Neutralization test results of sera and ascitic fluids from mice immunized with Cache Valley virus

Mouse*	Antibody titer	Day after initial immunization
1	<40	5
	640	20
8	<40	5
	160	20

*See Table 23.

TABLE 23. IgG and IgM antibody titers detected by ELISA from mice immunized with Cache Valley virus

		IgG and IgM titers after initial immunization			Booster immunization	
Mouse	globulin	day 5	day 10	day 21	Route	Day
1	IgG	2000	10000	3200	intrasplenic	5, 10
	IgM	800	<100	200		
2	IgG	2000	3200	3200	intrasplenic	5
	IgM	800	<100	<100		
3	IgG	400	1600	NT*	intrasplenic	5
	IgM	100	<100	NT		
4	IgG	800	1600	100	intrasplenic	none
	IgM	100	<100	<100		
5	IgG	800	1600	NT	intrasplenic	none
	IgM	800	800	NT		
6	IgG	800	800	NT	intrasplenic	none
	IgM	800	400	NT		
7	IgG	100	NT	NT	intraperitoneal	none
	IgM	<100	NT	NT		
8	IgG	400	NT	2000	intraperitoneal	5, 10
	IgM	<200	NT	<100		
9	IgG	400	NT	NT	intraperitoneal	5
	IgM	100	NT	NT		

*NT = not tested.

RNA-RNA BLOT HYBRIDIZATION

Genetic Relatedness of Eubenangee, Wallal, and Warrego Serogroup Viruses (H. A. Gonzalez and D. L. Knudson). Many of the recognized serogroups contain isolates from several geographic regions. For example, the Corriparta viruses have been isolated in Australia, South America, and Africa (Karabatsos et al., 1985). The viruses of the Eubenangee, Wallal, and Warrego serogroups were isolated in Australia with one exception, Pata.

The Eubenangee serogroup includes Eubenangee In1074 which was isolated in 1963 from a mixed pool of 11 mosquito species (Doherty et al., 1968), and a number of Australian viruses isolated from Culicoides midges, Anopheles mosquitoes, and Culex mosquitoes (Gard et al., 1973; Standfast et al., 1984). Pata virus was isolated from Aedes annulipes mosquitoes collected in the Central African Republic in 1968 (Karabatsos et al., 1985). Pata exhibits a low level of cross-reactivity with Eubenangee and Tilligerry in CF tests (Borden et al., 1971; Marshall et al., 1980). Eubenangee, Tilligerry, and Pata are distinct serotypes within the Eubenangee serogroup (Marshall et al., 1980).

Viruses in the Wallal and Warrego serogroups have been isolated only in Australia. The prototypes of these groups, Wallal Ch12048 and Warrego Ch9935, were isolated in Queensland from Culicoides midges (Doherty et al., 1973). Viruses which are serologically related to Wallal have been isolated from C. dycei and C. marksi (Doherty et al., 1973; Standfast et al., 1984). A second serotype, Mudjinbarry, was isolated from C. marksi collected in the Northern Territory (Doherty et al., 1978). The Warrego serogroup viruses have been isolated from Culicoides midges, Anopheles mosquitoes, and Culex mosquitoes (Doherty et al., 1973; 1979; Standfast et al., 1984). Mitchell River virus exhibits a low level of CF cross-reactivity with the Warrego prototype, and Mitchell River is distinguishable from Warrego in neutralization tests (Borden et al., 1971; Doherty et al., 1973). Although Mitchell River has not been re-isolated in Australia, multiple isolations of Wallal and Warrego viruses have been made (T. D. St. George, personal communication).

Viruses in these three serogroups exhibit characteristics typical of orbiviruses. They are acid labile, resistant to inactivation by detergents and solvents, and morphologically similar to other orbiviruses (Schnagl et al., 1969; Schnagl and Holmes, 1971; 1975; Gorman et al.,1978). One Eubenangee isolate has been shown to replicate in laboratory-infected Culicoides midges (Mellor and Jennings, 1980); and Eubenangee, Wallal, Warrego, and Mitchell River viruses have been shown to replicate in laboratory-infected mosquitoes (Carley et al., 1973). The viruses in these three serogroups have not been associated with human or animal illness, nor have they been isolated from vertebrates. Neutralizing antibody from marsupials and cattle to each of the three prototypes, however, has been reported (Doherty et al., 1970; Doherty et al., 1973).

Each serogroup contains several isolates, and the taxonomic status of all members within a serogroup has not been resolved by serologic tests. Three serotypes have been recognized in the Eubenangee serogroup, and the

Wallal and Warrego serogroups each have two serotypes. Two of the recognized serotypes, Pata and Mitchell River, exhibit low levels of CF cross-reactivity with their respective serogroup members. The remaining viruses have not been examined by neutralization tests.

The taxonomic status of these viruses is complicated by a low level serologic cross-reactivity between isolates in the Eubenangee and bluetongue serogroups. The Eubenangee and EHD viruses have been considered part of a bluetongue supergroup (Borden et al., 1971; Della-Porta, 1985; Gorman et al., 1985). However, cross-reactivity between the Wallal or Warrego viruses and bluetongue or EHD has not been reported.

RNA-RNA blot hybridization has been employed to examine the genetic relatedness of viruses in several other Orbivirus serogroups (Bodkin and Knudson, 1985b; 1986; Gonzalez and Knudson, 1987a; Brown et al., 1987 companion report). In this study, isolates from the Eubenangee, Wallal, and Warrego serogroups; bluetongue type 10; epizootic hemorrhagic disease (EHD 2); and Corriparta (MRM1) are examined by polyacrylamide and agarose gel electrophoresis and by RNA-RNA blot hybridization of gel-transfer images to determine the extent of genetic relatedness by genomic segment (Table 24).

Polyacrylamide gel electrophoresis. When the dsRNA genomes of viruses in the Eubenangee, Wallal, and Warrego serogroups were electrophoresed in polyacrylamide gels, most of the isolates exhibited distinctive profiles (Figs. 3 and 4). Four of the Eubenangee isolates had identical polyacrylamide gel profiles, while the remaining four were unique (Fig. 3). The Wallal isolates showed the least profile heterogeneity when compared to the other two serogroups. However, segments 2, 3, 5, and 6 of Wallal serogroup viruses exhibited varying mobilities, such that the three isolates were distinct (Fig. 4). The original Wallal isolate Ch12048 contained two electrophoretic types, Wallal-1 and Wallal-2, which were cloned by plaque-purification. The three Warrego isolates had distinguishable gel profiles (Fig. 4). The profile heterogeneity in each serogroup suggested that there was sequence heterogeneity among cognate genes.

Figure 5 shows the polyacrylamide gel profiles of members of six Orbivirus serogroups. In general, the gel profiles of the isolates in the bluetongue, EHD, Eubenangee, Wallal, and Warrego serogroups were similar with a 3-3-3-1 electrophoretic pattern of segments. Only Corriparta virus exhibited a markedly different profile. Gorman et al. (1981) reported that Eubenangee viruses could not be distinguished easily from bluetongue type 20 by polyacrylamide gel electrophoresis.

Agarose gel electrophoresis. The results of the agarose gel electrophoresis (Figs. 6 and 7) demonstrate that the segment molecular weight of any isolate was similar to its cognates in other isolates, that is, the intra-serogroup agarose profiles were essentially identical. Although the inter-serogroup profiles of isolates were distinctive for each serogroup; bluetongue, EHD, Eubenangee, Wallal, and Warrego serogroup viruses exhibited a common pattern when compared with Corriparta. Thus, polyacrylamide gel electrophoresis was used to prepare the blots because the identity of different isolates was confirmed.

Blot hybridization. Reciprocal blot hybridizations demonstrated that the majority of the ten segments are conserved among members of a serogroup (Figs. 8, 9, and 10). Genes that were conserved among isolates exhibited dark signals, while genes that were variant exhibited light or no signals. Light signals indicated that the shared sequence homology approached the lower limit (74%) required for the formation of stable hybrids under these stringency conditions, T_m(RNA)-36. Absent signals represented genes that did not cross-hybridize and their homology was <74%. In a similar study of the Palyam serogroup viruses (Bodkin and Knudson, 1986), the majority of Palyam segments were conserved among all isolates in the serogroup, with genes 2 and 6 exhibiting several variants. Genes 2 and 6 of the Eubenangee, Wallal, and Warrego isolates also exhibited variants. A conservative estimate of genetic relatedness within each of the three serogroups based upon these hybridization data is presented in Table 24.

Eubenangee Serogroup Viruses. Eubenangee, Tilligerry, and the six CSIRO isolates were closely related in the majority of the ten segments (Fig. 8a to d, data not shown). Four isolates, CSIRO 32, CSIRO 33, CSIRO 34 and CSIRO 36, were indistinguishable by blot hybridization (Fig. 8d), and they may represent repeated isolations of the same virus (Table 24). Pata did not cross-hybridize with any of the other Eubenangee isolates, except in genes 3 and 9 (Fig. 8b and d, data not shown). Segment 2 exhibited five unique genes in the remaining Eubenangee serogroup isolates, and segment 6 exhibited four variant genes. Segments 1 and 10 also exhibited variant genes. The remaining genes were conserved within these isolates (Table 25).

Wallal Serogroup Viruses. The three Wallal isolates cross-hybridized strongly in the majority of the segments (Fig. 9c and d). Again, genes 2 and 6 exhibited variants. Wallal-1 and Wallal-2 shared eight conserved genes, and they cross-hybridized weakly in genes 2 and 6. These two cloned viruses were distinct by polyacrylamide gel electrophoresis and blot hybridization. CSIRO 44 cross-hybridized strongly with Wallal-1 and Wallal-2 in eight genes. Gene 2 of CSIRO 44 did not cross-hybridize to its cognate in the other isolates (Table 24).

Warrego Serogroup Viruses. Warrego and CSIRO 12 were highly related in eight of the ten genes (Fig. 9). Genes 2 and 6 were variant between these two viruses. The third member of the serogroup, Mitchell River, did not cross-hybridize with the other Warrego isolates except in genes 4, 8, and 9 (Fig. 9a and b, data not shown).

Pata and Mitchell River viruses. The lack of strong cross-hybridization between Pata and any other Eubenangee serogroup isolate suggests that Pata should not be included in this serogroup. The degree of cross-hybridization between Pata and the remaining Eubenangee serogroup isolates was comparable to that seen between bluetongue type 10 and isolates of the EHD serogroup (Brown et al., 1987 companion report). Similarly, the cross-hybridization between Mitchell River and the other Warrego isolates resembles that seen between isolates in different serogroups, rather than isolates in the same serogroup. The results of the blot hybridizations are consistent with the results of CF tests. Pata does not cross-react strongly with other Eubenangee isolates (Borden et al., 1971; Marshall et al., 1980), nor does Mitchell River cross-react strongly with Warrego (Borden et al., 1971; Doherty et al., 1973). These two

isolates should be considered as members of the ungrouped set of orbiviruses.

Genetic Relatedness of Eubenangee, Wallal, and Warrego Serogroup Viruses. The results of the blot hybridizations between members of different serogroups supported the current serogroup divisions. None of the viruses in these serogroups cross-hybridized strongly to viruses in any of the other two serogroups, or to bluetongue type 10, EHD 2, or Corriparta virus (Fig. 10). When bluetongue dsRNA was used to probe the blot (Fig. 10a), only EHD 2 exhibited any cross-hybridization. Tilligerry cross-hybridized weakly to genes 1, 4, 6, 7, and 9 of the Wallal isolates; to genes 1, 3, and 9 of the Warrego isolates; and to genes 1 and 3 of bluetongue type 10. Both the Wallal-1 and Warrego probes cross-hybridized weakly to gene 9 of the Eubenangee serogroup isolates. Corriparta virus does not cross-hybridize to bluetongue, EHD, Wallal, or Warrego under identical conditions (D.K. Bodkin, 1985). The level of cross-hybridization between isolates in different serogroups shown here is no greater than that demonstrated between viruses in the bluetongue and EHD serogroups (Brown et al., 1987.

Gene 2 exhibits the greatest number of variants in these three serogroups, and it may be correlated with the gene encoding the neutralization antigen. This correlation has been shown in the Palyam serogroup viruses (Bodkin and Knudson, 1986). The occurrence of unique genes may reflect the strong selection pressure of the vertebrate immune system. Viruses in the Corriparta serogroup are not exposed to a strong immunologic selective pressure, and they did not exhibit unique genes (Gonzalez and Knudson, 1987a). These data suggest that vertebrates may play an unrecognized, important role in the life cycle of the Eubenangee, Wallal, and Warrego serogroup viruses.

A large number of viruses in these three serogroups were isolated at Beatrice Hill, in the Northern Territory of Australia during a two year interval (Table 24), indicating that these viruses co-circulate in nature. Since they may infect the same insect vector and host species, the potential for genetic reassortment is present. However, the inter-serogroup relatedness of these viruses suggests that they are distinct genetically, and thus, the serogroups may be maintained in nature as separate gene pools. Low levels of sequence homology between genes of isolates in different serogroups may result from infrequent reassortment between serogroups, or may simply reflect common ancestry (Gorman et al., 1985). Thus far, only intra-serogroup reassortment has been reported (Gorman et al., 1978; Kahlon et al., 1983), and the results of the reciprocal hybridizations suggest that inter-serogroup reassortment between viruses of the Eubenangee, Wallal, and Warrego serogroups is unlikely to occur. Experiments are in progress to correlate these hybridization data with biological reassortment.

RNA-RNA blot hybridization is a useful method of determining the genetic relatedness of orbiviruses. At least eight potentially distinct gene pools have been identified by RNA-RNA hybridization among isolates in Australia: Eubenangee, Wallal, Warrego, Mitchell River, bluetongue, EHD, Palyam, and Corriparta. If the viral gene pool were equated with the taxonomic level of serogroup, then common features emerge. For example, unique isolates within a gene pool have similar agarose gel profiles, but

distinct polyacrylamide gel profiles. They exhibit high CF cross-reactivity, and they cross-hybridize in the majority of the ten genes.

Serologic tests are useful predictors of relatedness, but interpretations of low level CF cross-reactivities as significant may lead to inconsistencies in the classification of orbiviruses. Orbivirus serogroups or gene pools should be defined as isolates which exhibit high CF cross-reactivity, high levels of sequence conservation, and an ability to reassort cognate genes.

Table 24. Eubenangee, Wallal, and Warrego Serogroup Viruses

Virus[a] Isolate	Source	Geographical Origin	Date of Collection
Eubenangee Serogroup			
Eubenangee In1074	Mosquito pool 11 species	Innisfail North Queensland Australia	16 Oct 63
Pata[b] Dak Ar B1327	Aedes palpalis	Pata Central African Republic	26 Nov 68
Tilligerry NB7080	Anopheles annulipes	Nelson Bay New South Wales Australia	22 Apr 71
- CSIRO 20	Culicoides marksi	Beatrice Hill Northern Territory Australia	14 May 75
- CSIRO 23	Anopheles farauti	Beatrice Hill Northern Territory Australia	2 Apr 75
- CSIRO 32	Culex annulirostris	Beatrice Hill Northern Territory Australia	26 Mar 75
- CSIRO 33	Culex annulirostris	Beatrice Hill Northern Territory Australia	2 Apr 75
- CSIRO 34	Culex annulirostris	Beatrice Hill Northern Territory Australia	26 Feb 75
- CSIRO 36	Culex annulirostris	Beatrice Hill Northern Territory Australia	29 Jan 75
Wallal Serogroup			
Wallal[c] Ch12048	Culicoides dycei	Charleville Queensland Australia	22 Feb 70
- CSIRO 44	Culicoides marksi	Beatrice Hill Northern Territory Australia	18 Dec 74
Warrego Serogroup			
Warrego Ch9935	Culicoides spp.	Charleville Queensland Australia	13 Feb 69

Table 24. (cont.) Eubenangee, Wallal, and Warrego Serogroup Viruses

Virus[a] Isolate	Source	Geographical Origin	Date of Collection
Mitchell River[b] Ch10434	Culicoides spp.	Charleville Queensland Australia	8 Apr 69
- CSIRO 12	Culicoides marksi	Beatrice Hill Northern Territory Australia	21 Nov 74

[a]Dash in the virus column indicates that a virus name has not been designated.

[b]The taxonomic status of Pata and Mitchell River should be reviewed (see text for discussion), and in the interim, the viruses should be placed in the ungrouped set of orbiviruses.

[c]Contained two isolates with different electropherotypes that were plaque-purified and are referred to as Wallal-1 and Wallal-2.

Table 25. Genetic Relatedness of Eubenangee, Wallal and Warrego Viruses[a]

Virus (Abbreviation) Isolate	Segment 1	2	3	4	5	6	7	8	9	10
Eubenangee Serogroup										
Eubenangee (EUB)										
In1074	eub	EUB	ebsg	ebsg	ebsg	eub	ebsg	ebsg	ebsg	eub
Tilligerry (TIL)										
NB7080	til	TIL	ebsg	ebsg	ebsg	til	ebsg	ebsg	ebsg	til
- CSIRO 20	til	CS20	ebsg	ebsg	ebsg	cs20	ebsg	ebsg	ebsg	eub
- CSIRO 23	til	CS23	ebsg	ebsg	ebsg	cs23	ebsg	ebsg	ebsg	eub
- CSIRO 32	til	CS32	ebsg	ebsg	ebsg	eub	ebsg	ebsg	ebsg	eub
- CSIRO 33	til	CS32	ebsg	ebsg	ebsg	eub	ebsg	ebsg	ebsg	eub
- CSIRO 34	til	CS32	ebsg	ebsg	ebsg	eub	ebsg	ebsg	ebsg	eub
- CSIRO 36	til	CS32	ebsg	ebsg	ebsg	eub	ebsg	ebsg	ebsg	eub
Wallal Serogroup										
Wallal-1 (WAL)										
Ch12048	wlsg	WAL1[b]	wlsg	wlsg	wlsg	wal1	wlsg	wlsg	wlsg	wlsg
Wallal-2 (WAL)[c]										
Ch12048	wlsg	WAL2[b]	wlsg	wlsg	wlsg	wal2	wlsg	wlsg	wlsg	wlsg
- CSIRO 44	wlsg	CS44	wlsg	wlsg	wlsg	cs44	wlsg	wlsg	wlsg	wlsg

Table 25(continued). Genetic Relatedness of Eubenangee, Wallal and Warrego Viruses[a]

Virus (Abbreviation) Isolate	Segment 1	2	3	4	5	6	7	8	9	10
Warrego Serogroup										
Warrego (WAR)										
Ch9935	wrsg	WAR	wrsg	wrsg	wrsg	war	wrsg	wrsg	wrsg	wrsg
-										
CSIRO 12	wrsg	CS12	wrsg	wrsg	wrsg	cs12	wrsg	wrsg	wrsg	wrsg
Ungrouped Isolates										
Pata (PATA)[d]										
Dak Ar B1327	PATA	PATA	pata	PATA	PATA	PATA	PATA	PATA	pata	PATA
Mitchell River (MR)[d]										
Ch10434	MR	MR	MR	mr	MR	MR	MR	mr	mr	MR

[a]The estimates of genetic relatedness were made following the convention of Bodkin and Knudson (1985b; 1986). Serogroup genes, which cross-hybridized strongly in all isolates, are designated by ebsg (Eubenangee serogroup gene), wlsg (Wallal serogroup gene), or wrsg (Warrego serogroup gene). Variant genes that differed in degree of hybridization to cognates of heterologous isolates are indicated by a lower case abbreviation of the name of an isolate. When variant genes of two or more viruses cross-hybridized strongly, the genes were arbitrarily assigned the designation of one of the viruses involved. Unique genes, which did not hybridize to cognate genes of heterologous isolates, are indicated by an upper case abbreviation of the name of an isolate.

[b]Gene 2 of Wallal-1 and Wallal-2 were unique with respect to CSIRO 44 and variant with respect to each other.

[c]Segments are referred to according to their cognates in the prototype viruses. Since the third segment from the top of the dsRNA profiles of Wallal-2 and CSIRO 12 cross-hybridized to the second segment of Wallal-1 and Warrego, respectively, they are referred to as gene 2. Likewise, the second segment from the top of the gel in these isolates is referred to as gene 3.

[d]Genes 3 and 9 of Pata cross-hybridized weakly to cognates in the Eubenangee serogroup viruses. Genes 4, 8, and 9 of Mitchell River cross-hybridized weakly to cognates in the Warrego serogroup viruses. These two isolates were considered to be ungrouped on the basis of these data.

Figure 3. Polyacrylamide gel depicting the resolution of the dsRNA genomes of nine Eubenangee serogroup isolates. Genomic RNA was electrophoresed through a Tris-glycine buffered 10% polyacrylamide gel. The gel was stained with ethidium bromide (0.5 ug/ml) for 24 h. Lanes are from left to right uninfected cellular control (Lane 1), reovirus type 3 (Lane 2), Eubenangee (Lane 3), Tilligerry (Lane 4), Pata (Lane 5), CSIRO 20 (Lane 6), CSIRO 23 (Lane 7), CSIRO 32 (Lane 8), CSIRO 33 (Lane 9), CSIRO 34 (Lane 10), and CSIRO 36 (Lane 11). Segments are numbered 1 to 10 from the top of the gel.

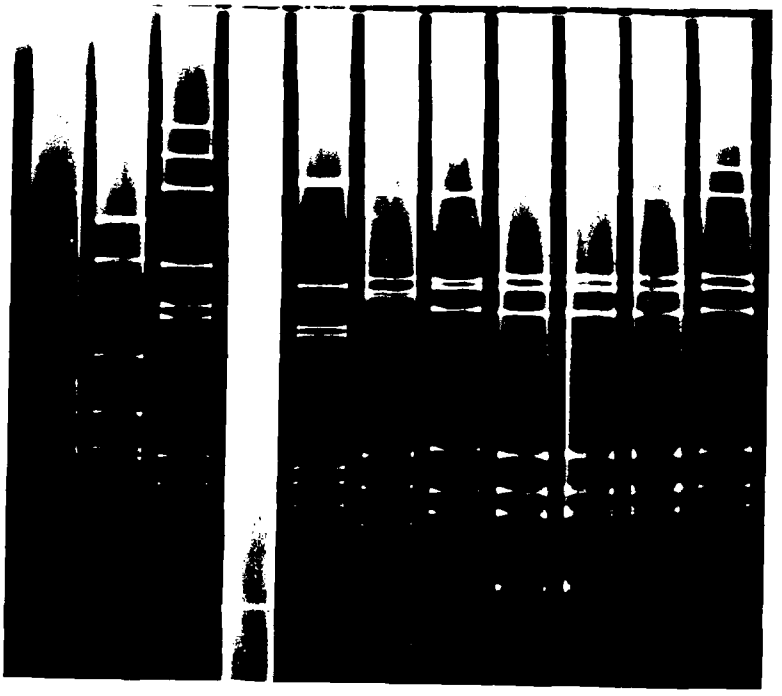


Figure 4. Polyacrylamide gel depicting the resolution of the dsRNA genomes of three Warrego and three Wallal serogroup isolates. Lanes are from left to right uninfected cellular control (Lane 1), reovirus type 3 (Lane 2), Warrego (Lane 3), Mitchell River (Lane 4), CSIRO 12 (Lane 5), Wallal-1 (Lane 6), Wallal-2 (Lane 7), and CSIRO 33 (Lane 8).

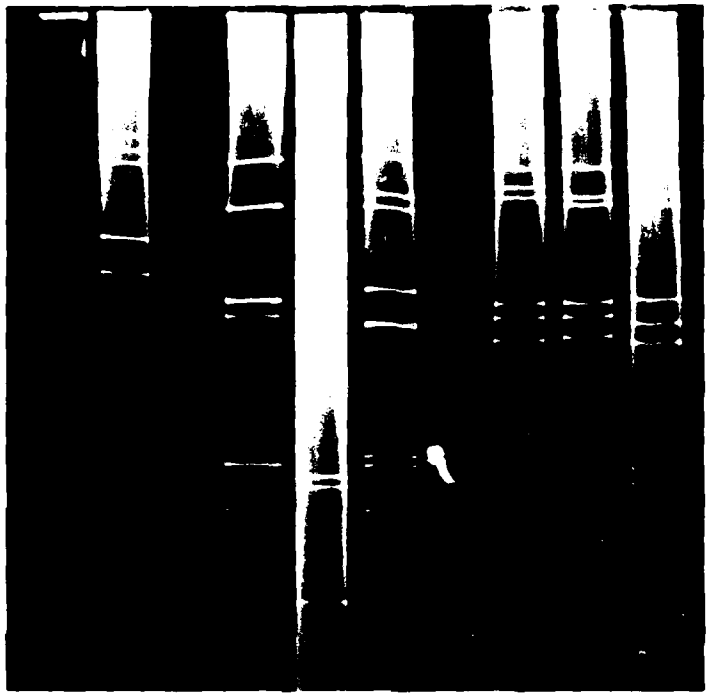


Figure 5. Polyacrylamide gel depicting the resolution of dsRNA genomes of orbiviruses of several different serogroups. Lanes are from left to right uninfected cellular control (Lane 1), reovirus type 3 (Lane 2), bluetongue type 10 (Lane 3), EHD 2 (Lane 4), Eubenangee (Lane 5), Tilligerry (Lane 6), CSIRO 23 (Lane 7), Warrego (Lane 8), CSIRO 12 (Lane 9), Wallal-1 (Lane 10), Wallal-2 (Lane 11), and Corriparta (Lane 12).

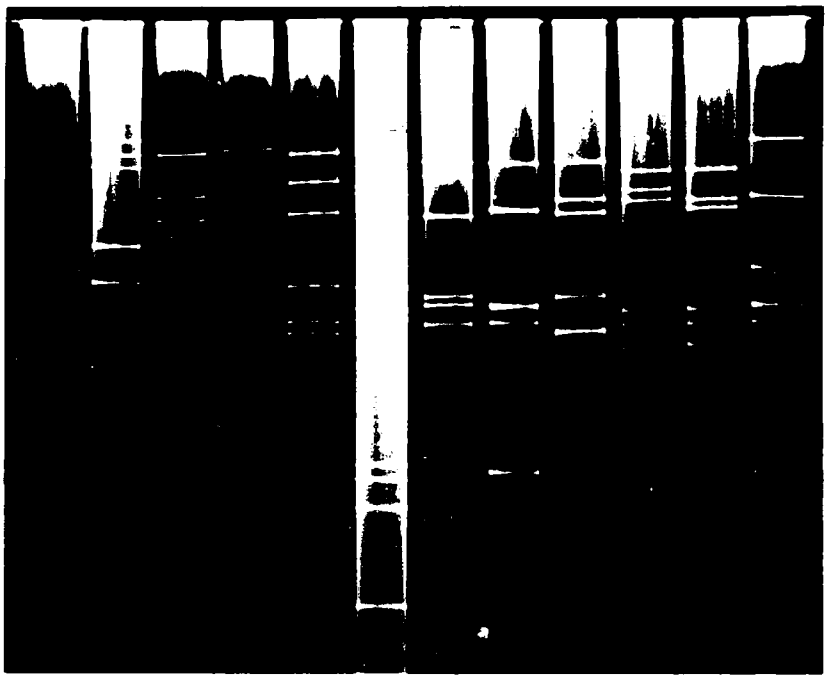


Figure 6. Autoradiogram depicting the resolution of the dsRNA genomes of the Eubenangee isolates by electrophoresis through a 1% agarose gel. Lanes are from left to right reovirus type 3 (Lane 1), Eubenangee (Lane 2), Tilligerry (Lane 3), CSIRO 20 (Lane 4), CSIRO 23 (Lane 5), CSIRO 32 (Lane 6), CSIRO 33 (Lane 7), CSIRO 34 (Lane 8), CSIRO 34 (Lane 8), CSIRO 36 (Lane 9), Pata (Lane 10), and EHD 2 (Lane 11).

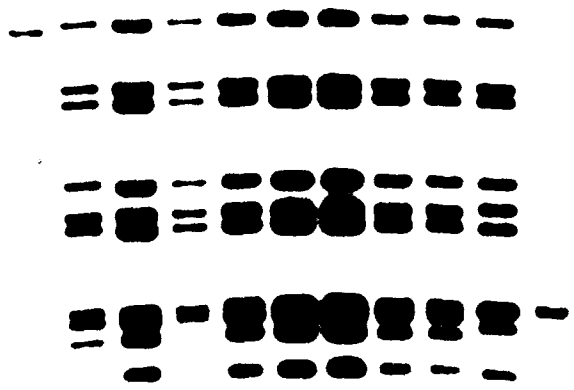


Figure 7. Autoradiogram depicting the resolution of the dsRNA genomes of orbiviruses of several different serogroups. Lanes are from left to right reovirus type 3 (Lane 1), bluetongue type 10 (Lane 2), EHD 2 (Lane 3), Eubenangee (Lane 4), Tilligerry (Lane 5), CSIRO 23 (Lane 6), Warrego (Lane 7), CSIRO 12 (Lane 8), Mitchell River (Lane 9), Wallal-1 (Lane 10), Wallal-2 (Lane 11), CSIRO 44 (Lane 12), and Corriparta (Lane 13).

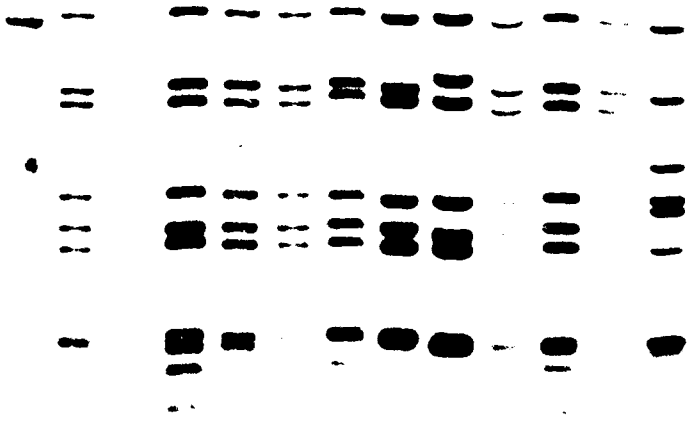
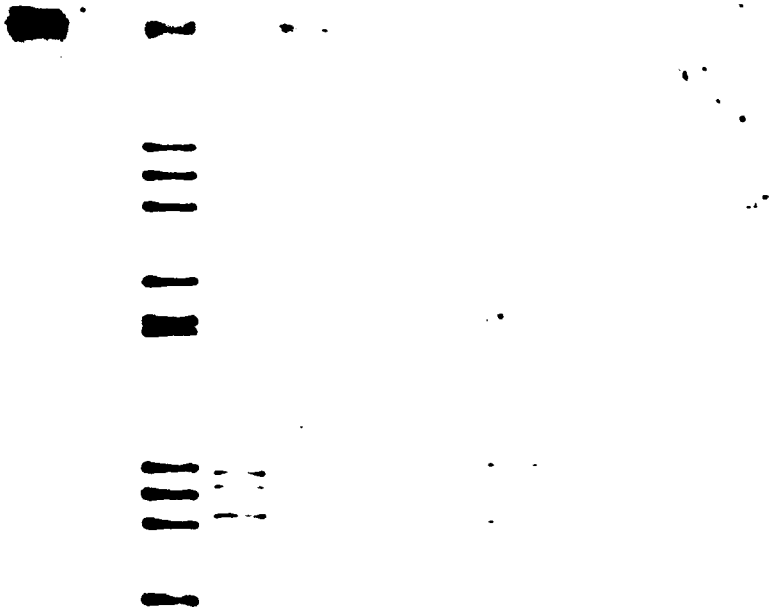
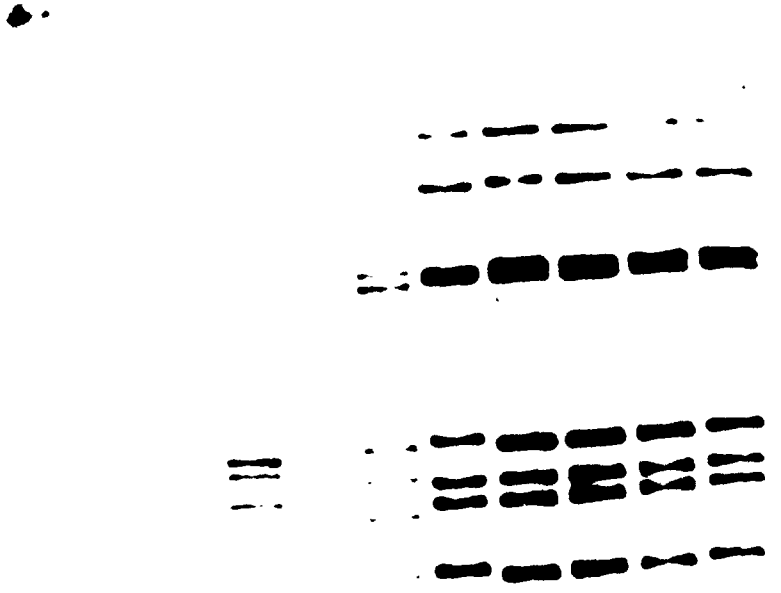


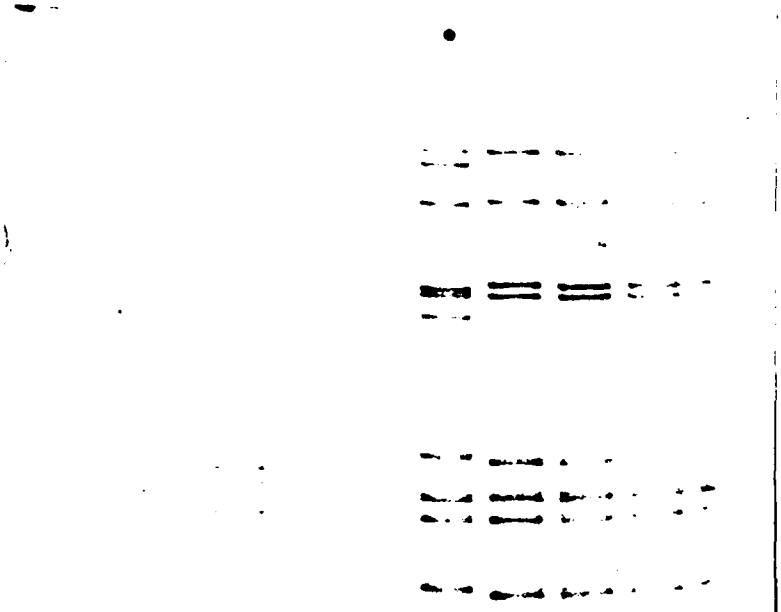
Figure 8. Autoradiograms depicting hybridization of probe genes to cognates in the Eubenangee serogroup viruses. Total genomic dsRNA was end-labelled with [5'-^{32}P]pCp and hybridized to the genomic profiles of the serogroup members after transfer from polyacrylamide gels to Zeta-Probe membrane. Lanes are designated as described in Figure 3. The membrane was hybridized with Eubenangee (Fig. 8a), CSIRO 20 (Fig. 8b), CSIRO 23 (Fig. 8c), and CSIRO 32 (Fig. 8d).



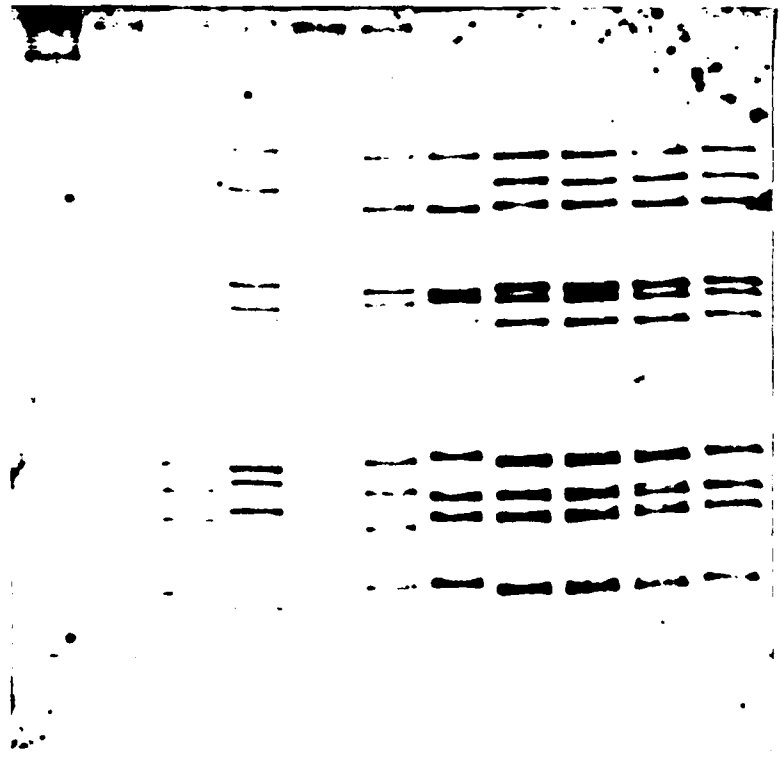
8a



8b

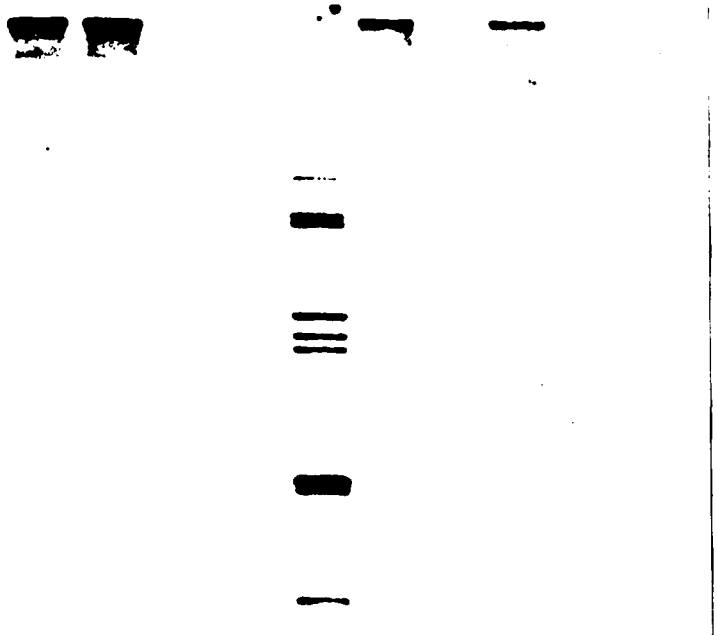


8c

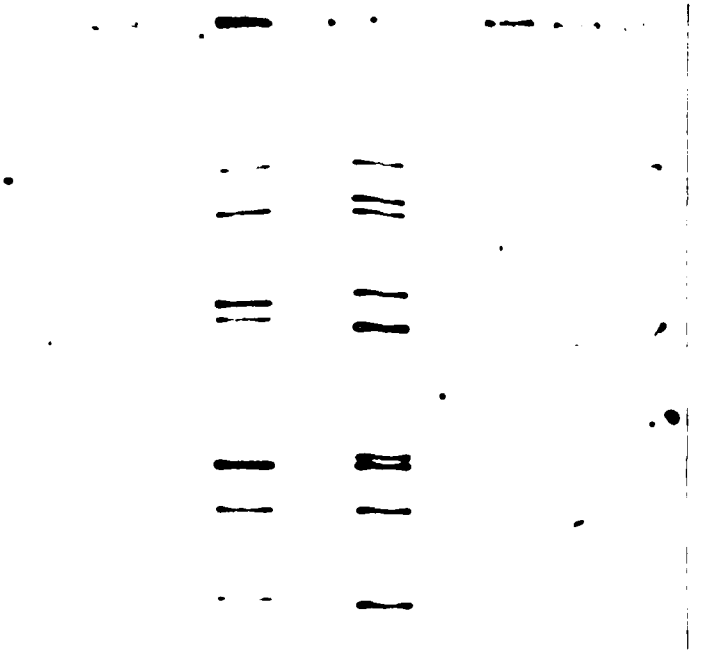


8d

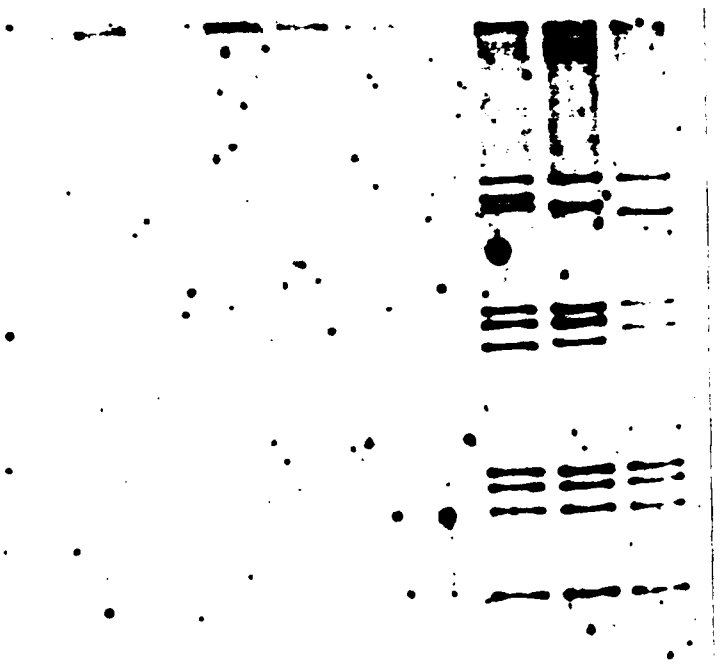
Figure 9. Autoradiograms depicting hybridization of probe genes to cognates in the Warrego and Wallal serogroup viruses as described in Figure . Lanes are designated as described in Figure 4. The membrane was hybridized with Mitchell River (Fig. 9a), CSIRO 12 (Fig. 9b), Wallal-1 (Fig. 9c), and Wallal-2 (Fig. 9d).



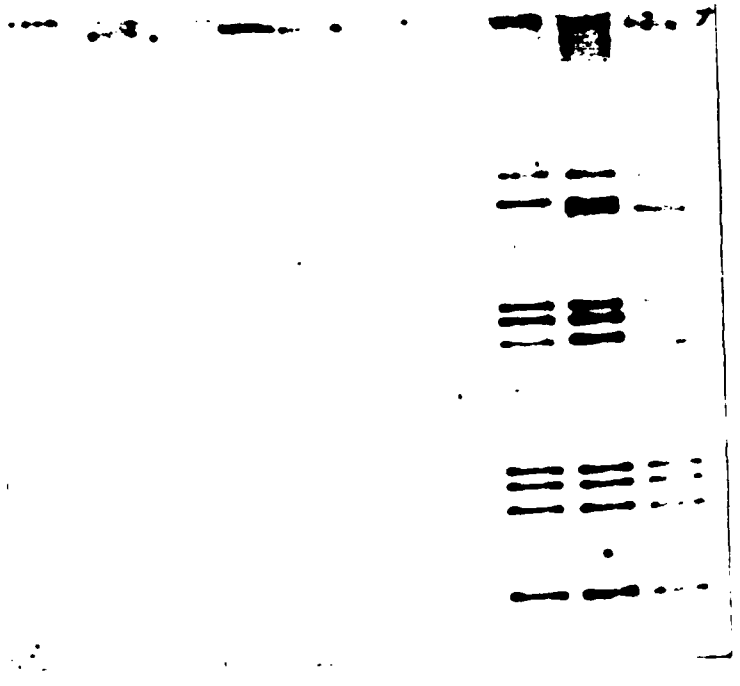
9a



9b

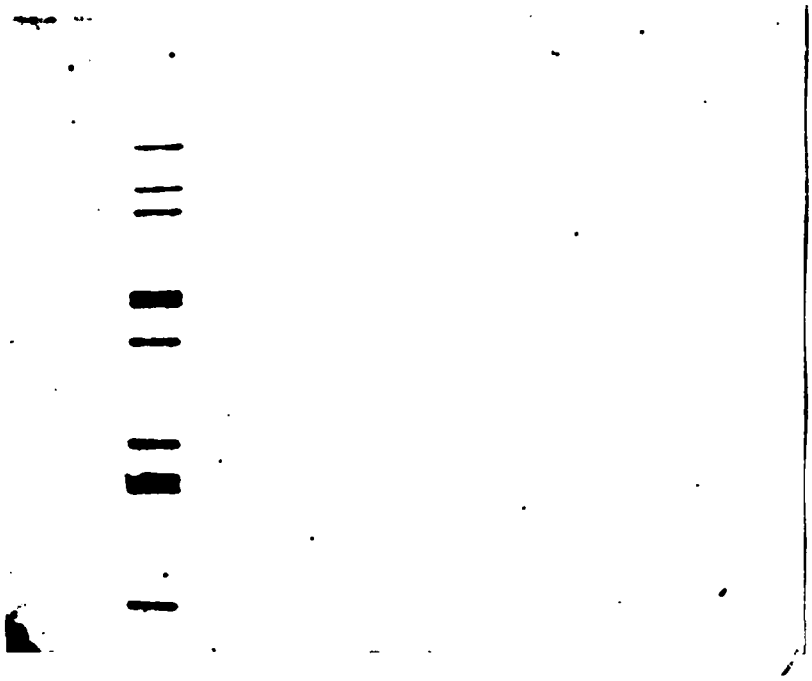


9c

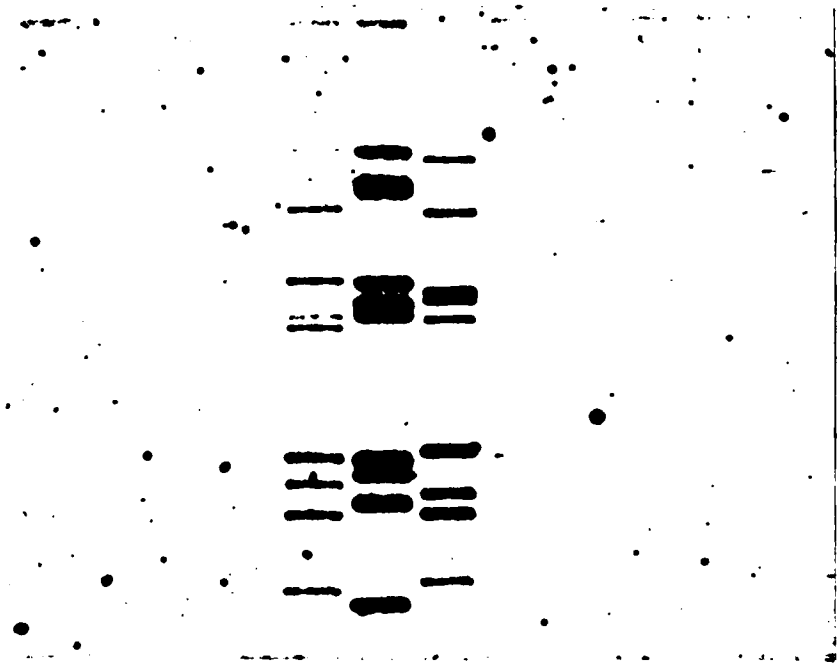


9d

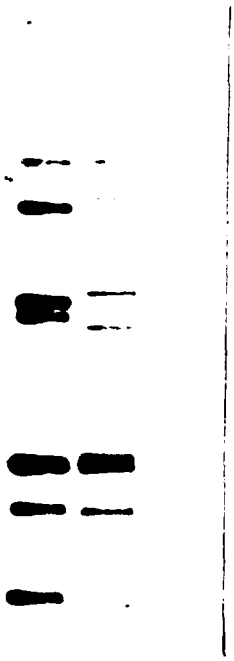
Figure 10. Autoradiograms depicting hybridization of probe genes to cognates in viruses in several orbivirus serogroups as described in Figure 8. Lanes are designated as described in Figure 5. The membrane was hybridized with bluetongue type 10 (Fig. 10a), Tilligerry (Fig. 10b), Warrego (Fig. 10c), and Wallal-1 (Fig. 10d).



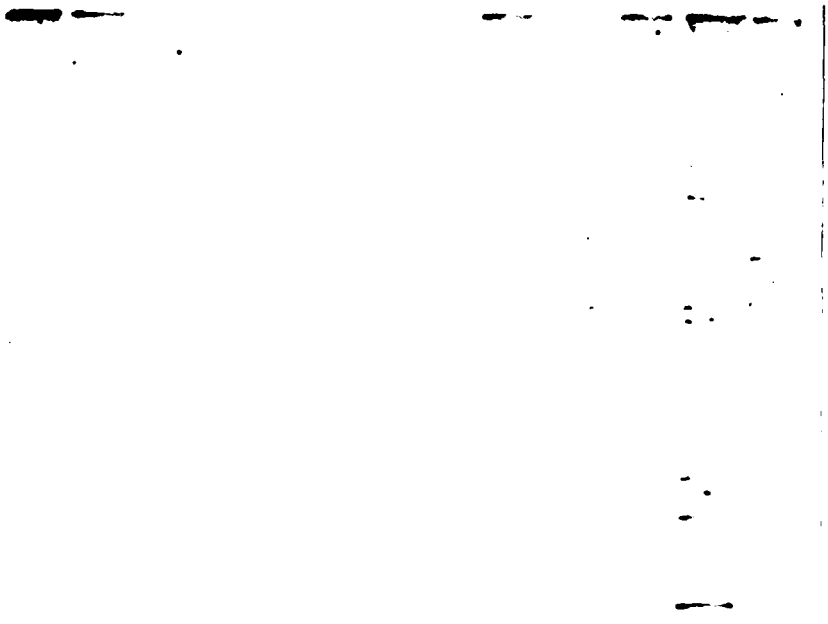
10a



10b



10c



10d

Genetic relatedness of Epizootic Hemorrhagic Disease Serogroup, Bluetongue Type 10, and Pata Viruses (S.E. Brown, D.K. Bodkin, R.B. Tesh and D.L. Knudson). Viruses which belong to the epizootic hemorrhagic disease (EHD), bluetongue, and Eubenangee serogroups cross-react in some serologic tests and have been regarded as a cluster of viruses. (Borden et al., 1971; Moore and Lee, 1972; Moore, 1974; Gorman and Taylor, 1978; Gorman, 1979). Although members of the EHD, bluetongue, and Eubenangee serogroups appear to be related, the biological characteristics of these viruses are different. EHD and bluetongue viruses cause disease in different animal hosts, deer and sheep respectively. Eubenangee viruses have not been associated with disease in animals (Gorman, 1979). EHD and bluetongue are vectored principally by Culicoides spp., whereas Eubenangee viruses have been isolated primarily from mosquitoes (Erasmus, 1985; Knudson and Shope, 1985; Gonzalez and Knudson, 1987b).

Pata was assigned to the Eubenangee serogroup even though Pata cross-reacts at a low level in complement-fixation tests with Eubenangee, EHD, and bluetongue (Borden et al., 1971; Gorman et al., 1983). However, RNA-RNA blot hybridization data demonstrated that Pata does not belong to the Eubenangee serogroup (Gonzalez and Knudson, 1987b).

In this study, the genetic relatedness among members of the EHD serogroup, bluetongue type 10 (BTV 10) (Knudson, et al., 1982), and Pata was assessed by RNA-RNA blot hybridization (Table 26). The EHD isolates examined were EHD 1 (Shope et al., 1955; 1960), EHD 2 (Knudson et al., 1982), IbAr 22619 (Lee et al., 1974), IbAr 33853 (Lee et al., 1974), and JKT-9133 (Tesh et al., 1986).

Members within orbivirus serogroups exhibit similar agarose profiles (Bodkin and Knudson, 1985b; Gonzalez and Knudson, 1987a; 1987b), while the agarose profiles of members from different orbivirus serogroups are distinguishable (Gonzalez and Knudson, 1987b). Four of the 5 EHD isolates had identical agarose profiles (Fig. 11). Genes 5 and 9 of the JKT-9133 isolate migrated faster in the gel than did genes 5 and 9 of the other EHD isolates. The significance of this minor difference in the JKT-9133 isolate is unknown. While similar, the agarose profiles of BTV 10 and Pata were distinct from each other and from the EHD isolates.

Isolates of an orbivirus serogroup which have similar agarose profiles may exhibit different PAGE profiles (Bodkin and Knudson, 1985b; Gonzalez and Knudson, 1987a; 1987b). The EHD isolates, BTV 10, and Pata exhibited unique PAGE profiles (Fig. 12). Gene 6 was comigrant in all the EHD isolates, and EHD 1 and IbAr 22619 exhibited similar PAGE profiles.

In this study, the segments were referred to according to their cognates in the prototype EHD 1. Cognate genes are genes which are functionally equivalent between different isolates (Gaillard and Joklik, 1982).

In reciprocal RNA-RNA blot hybridizations, highly conserved and variant genes among the different isolates were determined by the intensity of hybridization signals. Conserved genes exhibited dark signals, while variant genes exhibited light signals. The cross-hybridization of genes which results in light signals indicates that their shared sequences approach the lower limit of 74% homology required for the formation of

stable hybrids. At T_m(RNA)-36, unique genes or genes which did not form stable hybrids with each other were <74% homologous.

Genetic relatedness of the EHD serogroup. Reciprocal RNA-RNA blot hybridizations of the 5 EHD isolates demonstrated that the EHD isolates were ≥74% homologous in 9 of their 10 genes. Unique, variant, and conserved genes within the EHD serogroup were identified (Fig. 13). Gene 2 was unique to some of the EHD isolates, and genes 5 and 10 were variant. EHD 1 and IbAr 22619 were highly conserved in gene 2, and IbAr 33853 and JKT-9133 were variant in gene 2. There were 4 variant types of gene 5 and 2 variant types of gene 10.

EHD 1 and IbAr 22619 were more closely related to each other by PAGE profile and by hybridization than to the other EHD isolates. These two viruses shared ≥74% homology in all 10 genes. The rest of the EHD isolates showed lesser degrees of relatedness. Although JKT-9133 exhibited a distinctive agarose profile, JKT-9133 was an EHD isolate by hybridization (Fig. 13). A conservative estimate for the genetic relatedness of the EHD serogroup viruses based upon the reciprocal hybridizations is presented in Table 27.

Geographic boundaries could not be correlated with sequence relatedness between EHD isolates. EHD 1 and IbAr 22619 exhibited a high degree of relatedness by hybridization, but they were isolated from two different continents. Gene 10 of EHD 1 was variant when compared to gene 10 of IbAr 22619. Although IbAr 22619 and IbAr 33853 were isolated from the same locality 1 year apart, IbAr 22619 gene 2 was unique when compared to IBAr 33853 gene 2 and IbAr 22619 gene 5 was variant when compared to IbAr 33853 gene 5. Therefore, IbAr 22619 and IbAr 33853 were more distantly related to each other than EHD 1 and IbAr 22619.

The outer capsid of EHD 1 consists of 2 major proteins P2 and P5, and a minor protein P3A (Huismans et al., 1979). The EHD genes which encode the large surface antigens may be subjected to the strong evolutionary pressure of the vertebrate immune system which could result in sequence divergence in these genes. P2 and P5 may be encoded by genes 2 and 5 which exhibited sequence divergence among the EHD isolates. Gene 2 which is unique to some of the EHD isolates may encode the neutralization antigen. The hybridization data correlate with the following neutralization data to support this thesis. For example, EHD 1 and EHD 2 are distinct by plaque-reduction tests (Campbell et al., 1978), IbAr 22619 and IbAr 33853 are distinct by plaque-reduction tests (Campbell and St George, 1986), and EHD 1 and IbAr 22619 cross-react in cross-neutralization tests in suckling mice (Moore and Lee, 1972; Moore, 1974).

Genetic relatedness between the EHD serogroup, BTV 10, and Pata. RNA-RNA blot hybridization demonstrated that EHD serogroup viruses were distantly related to BTV 10 and Pata (Fig. 13). Highly conserved genes were not seen in inter-serogroup hybridization among the EHD isolates, BTV 10, and Pata. The relatedness of the genes among the EHD isolates, BTV 10, and Pata approached the lower limits of detectability under these stringency conditions.

The numbers of genes which appeared to cross-hybridize between BTV 10 and each of the EHD isolates were not always consistent in the reciprocal

hybridization reactions, except for gene 2 which was always unique to BTV 10. Gene 2 of BTV 10 was also unique when compared to gene 2 of Pata. Gene 2 of BTV 10 encodes P2 (Kahlon et al., 1983), and BTV P2 has been associated with BTV serotype-specificity (Huismans and Erasmus, 1981).

A BTV 10 gene hybridized to gene 9 of the EHD isolates more strongly than the other BTV 10 genes hybridized to their cognates in EHD (Fig. 13). While the function of EHD gene 9 is unknown, it could encode EHD P7 which shares antigenic determinants with BTV P7 (Huismans et al., 1979). The appearance and decline of BTV P7 precipitating antibodies in an infected sheep corresponded to the levels of complement-fixing antibodies which suggests that BTV 10 P7 is the protein which determines group-specificity (Huismans and Erasmus, 1981).

Genetic relatedness of orbivirus serogroups. Viruses within orbivirus serogroups (Bodkin and Knudson, 1985b; 1986; Gonzalez and Knudson, 1987a; 1987b), which cross-react strongly in CF tests are highly related by RNA blot hybridization in most of their genes. Likewise, viruses from different serogroups which do not cross-react strongly in CF tests are not highly related by RNA blot hybridization.

Although Pata cross-reacts in CF tests with members of the Eubenangee, bluetongue and EHD serogroups, these low level CF cross-reactivities must be viewed with caution. These hybridization data suggest that Pata is not a member of either the Eubenangee, bluetongue, or EHD serogroups (Gonzalez and Knudson, 1987b). Since Pata is not highly cross-reactive in CF tests with any known orbivirus serogroup, the genetic relatedness of Pata to the ungrouped set of orbiviruses must be assessed. Mitchell River represents another example of a virus which cross-reacts at low levels in serologic tests with the Warrego serogroup members, and an examination of intra-serogroup genetic relatedness suggests that Mitchell River may have been classified inappropriately (Gonzalez and Knudson, 1987b).

The currently recognized Kemerovo serogroup viruses also contain numerous examples of low-level CF cross-reactivities between selected members (Borden et al., 1971), and these findings have prompted the use of "serocomplex" as a subgrouping term (Casals, 1971; Libikova and Casals, 1971). While hybridization data from this laboratory confirm the earlier recognition of subgroupings within the Kemerovo serogroup viruses, the data also suggest that the taxonomic status of these viruses may be represented as several distinct serogroups (Brown and Knudson, manuscript in preparation and unpublished results).

The currently recognized Kemerovo serogroup viruses also contain numerous examples of low-level CF cross-reactivities between selected members (Borden et al., 1971), and these findings have prompted the use of "serocomplex" as a subgrouping term (Main, personal communication). The results from hybridization experiments in this laboratory indicate that the taxonomic status of the Kemerovo serogroup viruses may also require revision (Brown and Knudson, manuscript in preparation and unpublished results).

Thus, the EHD isolates exhibited similar agarose profiles, and they were related highly by RNA-RNA blot hybridization. Unique, variant, highly conserved EHD genes were identified, EHD genes which may be associated with EHD serogroup- and serotype-specificity were also identified. The EHD

isolates, BTV 10, and Pata cross-react at low levels in serological tests (Borden et al., 1971), exhibit distinct agarose profiles, and are related distantly by RNA-RNA blot hybridization. These data suggest that in nature these viruses are maintained as distinct gene pools, and their distant relatedness may reflect a common ancestry. Gene reassortment experiments are in progress in this laboratory to determine the potential for these viruses to interact genetically.

Table 26

Virus Isolates

Virus	Strain	Isolation Source	Geographical Origin	Year[a]
EHD 1	New Jersey	Deer	Morris County, New Jersey USA	1955
EHD 2	Can Alberta	Deer	Alberta, Canada	1962
	IbAr 22619	Culicoides spp.	Ibadan, Nigeria	1967
	IbAr 33853	Culicoides spp.	Ibadan, Nigeria	1968
	JKT-9133	Anopheles vagus	Tag-Tag, Bali Indonesia	1981
Bluetongue Type 10	BT 8	sheep	United States	1953
Pata[b]	DakArB 1327	Aedes palpalis	Central African Republic	1968

[a]Represents the year in which the isolate was collected in the field.

[b]The taxonomic status of Pata should be reviewed (see text for discussion), and in the interim, it should be placed in the ungrouped set of orbiviruses.

Table 27

Genetic Relatedness of EHD Serogroup Viruses[a]

Isolate	Genes									
	1	2	3	4	5	6	7	8	9	10
EHD 1	ehsg[b]	EH1[c]	ehsg	ehsg	ehld	ehsg	ehsg	ehsg	ehsg	ehl
EHD 2	ehsg	EH2	ehsg	ehsg	eh2	ehsg	ehsg	ehsg	ehsg	ehl
Ib An 22619	ehsg	EH1	ehsg	ehsg	ehl	ehsg	ehsg	ehsg	ehsg	ib3
Ib An 33853	ehsg	IB3[e]	ehsg	ehsg	ib3	ehsg	ehsg	ehsg	ehsg	ib3
JKT 9133	ehsg	JKT[e]	ehsg	ehsg	jkt	ehsg	ehsg	ehsg	ehsg	ib3

[a]Membranes containing the genome profiles of each of the isolates were hybridized to 1 ug of [5'-^{32}P] pCp-labelled genomic RNA from each of the other isolates as described in the text.

[b]Conserved genes exhibited dark signals and were designated ehsg (EHD serogroup gene)

[c]Genes which did not cross-hybridize to their cognates were designated by the virus name in upper case. If two viruses cross-reacted strongly to each other and they were unique to the other isolates, the gene was identified by the name of the earliest isolate in upper case

[d]Variant genes hybridized weakly to their cognates when they were used as probes, and they were designated by the name of the earliest isolate where necessary in lower case letters.

[e]These two viruses were unique in this gene with respect to the remaining viruses, but they were variant to each other in this gene.

Agarose Gel Profiles of EHD, BTV, and Pata

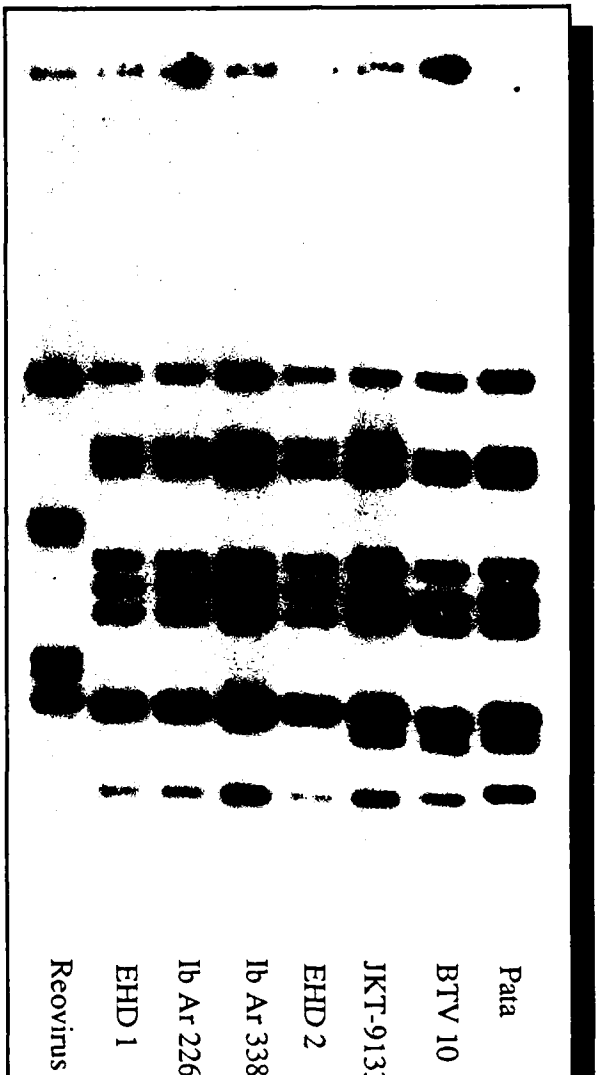


Figure 11. Autoradiogram depicting the profiles of the EHD serogroup isolates, bluetongue type 10, and Pata in 1% agarose. Genomic RNA was end-labelled with [5'-^{32}P]pCp and electrophoresed through a 1% agarose gel. Lanes are from left to right reovirus 3 Dearing strain (Lane 1), EHD 1 (Lane 2), IbAr 22619 (Lane 3), IbAr 33853 (Lane 4), EHD 2 (Lane 5), JKT-9133 (Lane 6), BTV 10 (Lane 7), and Pata (Lane 8).

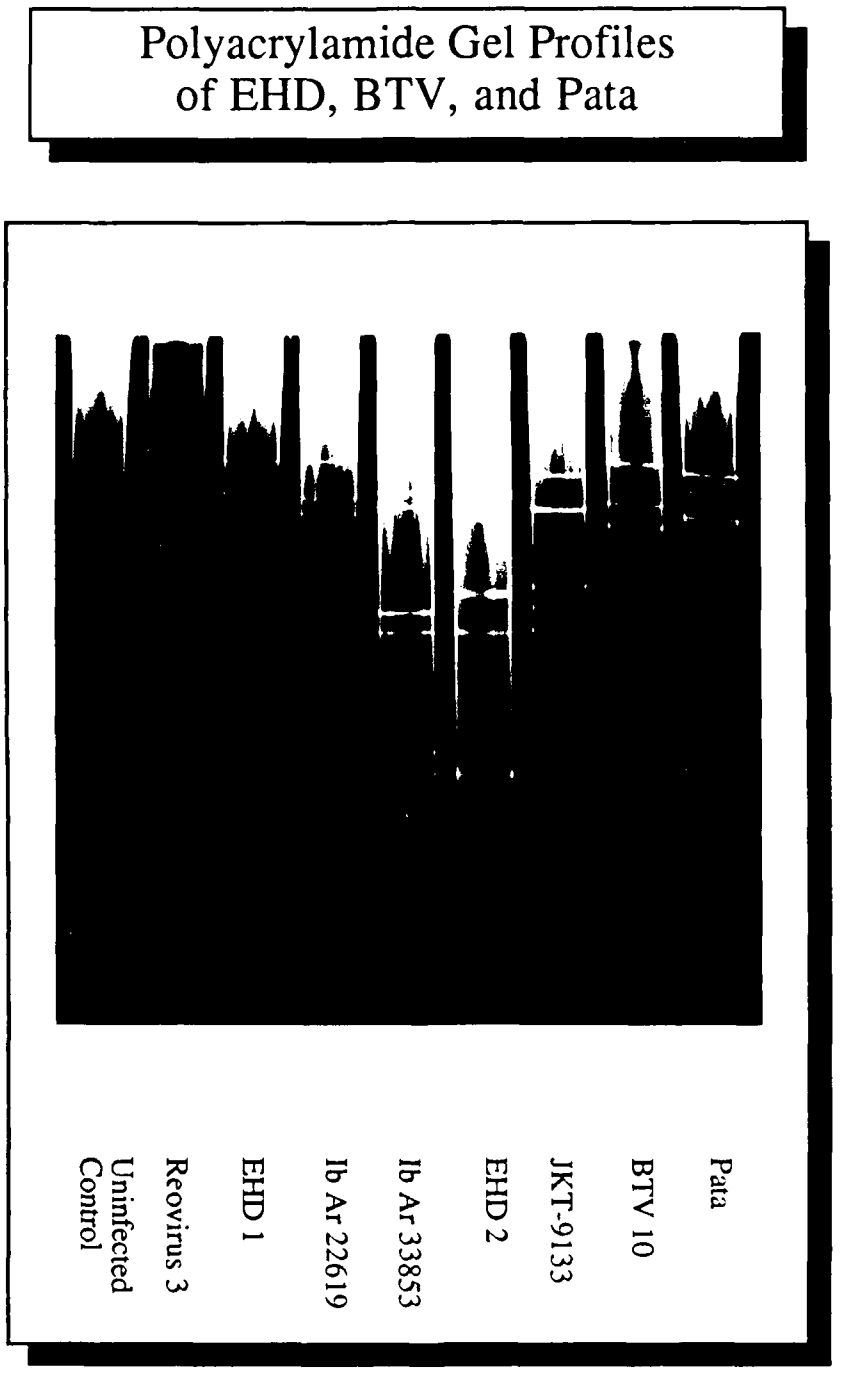


Figure 12. The dsRNA profiles of the EHD serogroup isolates, bluetongue type 10, and Pata in 10% polyacrylamide gel stained with ethidium bromide. Lanes are from left to right uninfected control (Lane 1), reovirus 3 Dearing strain (Lane 2), EHD 1 (Lane 3), IbAr 22619 (Lane 4), IbAr 33853 (Lane 5), EHD 2 (Lane 6), JKT-9133 (Lane 7), BTV 10 (Lane 8), and Pata (Lane 9). Segments are number 1 to 10 from the top of the gel.

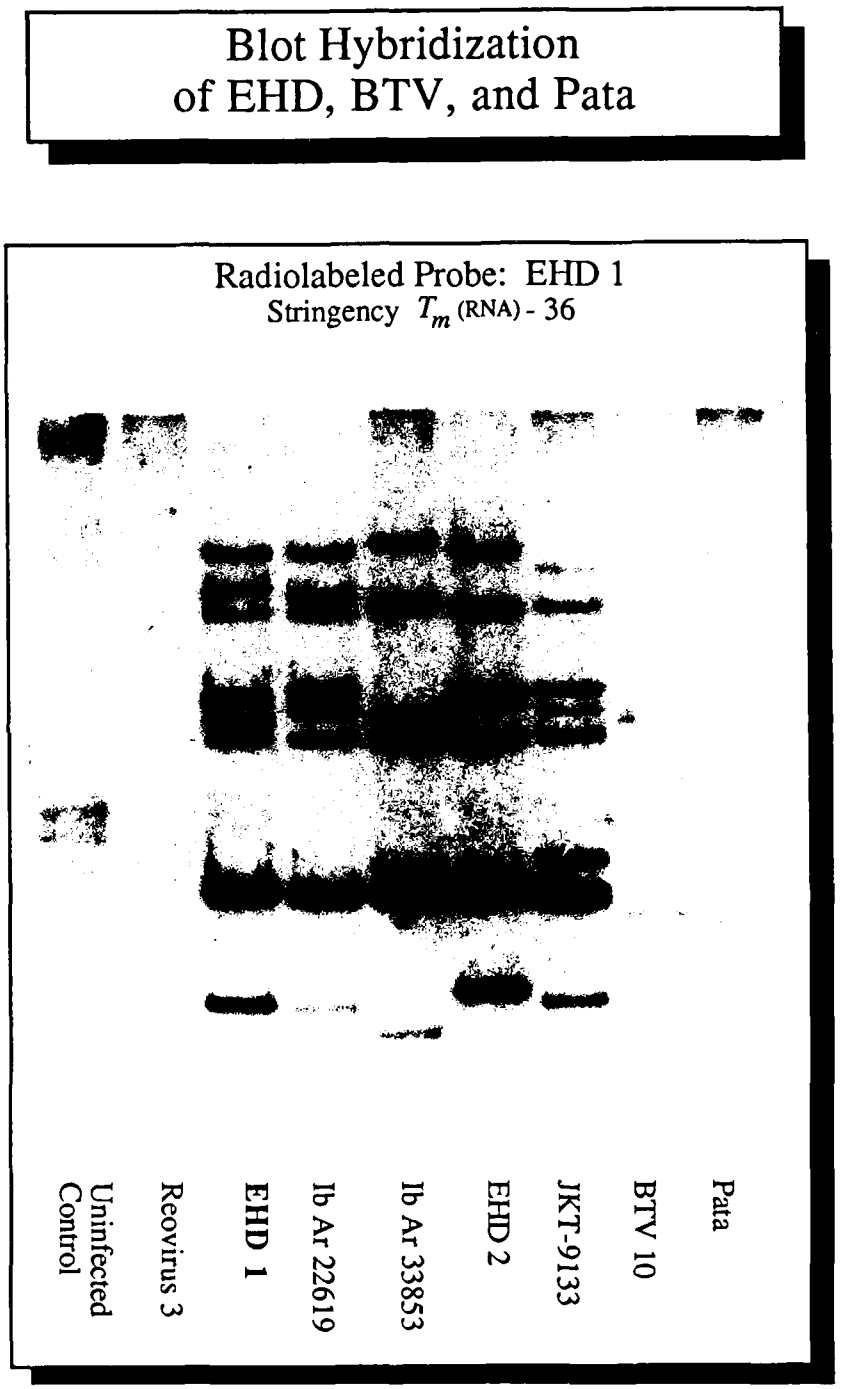
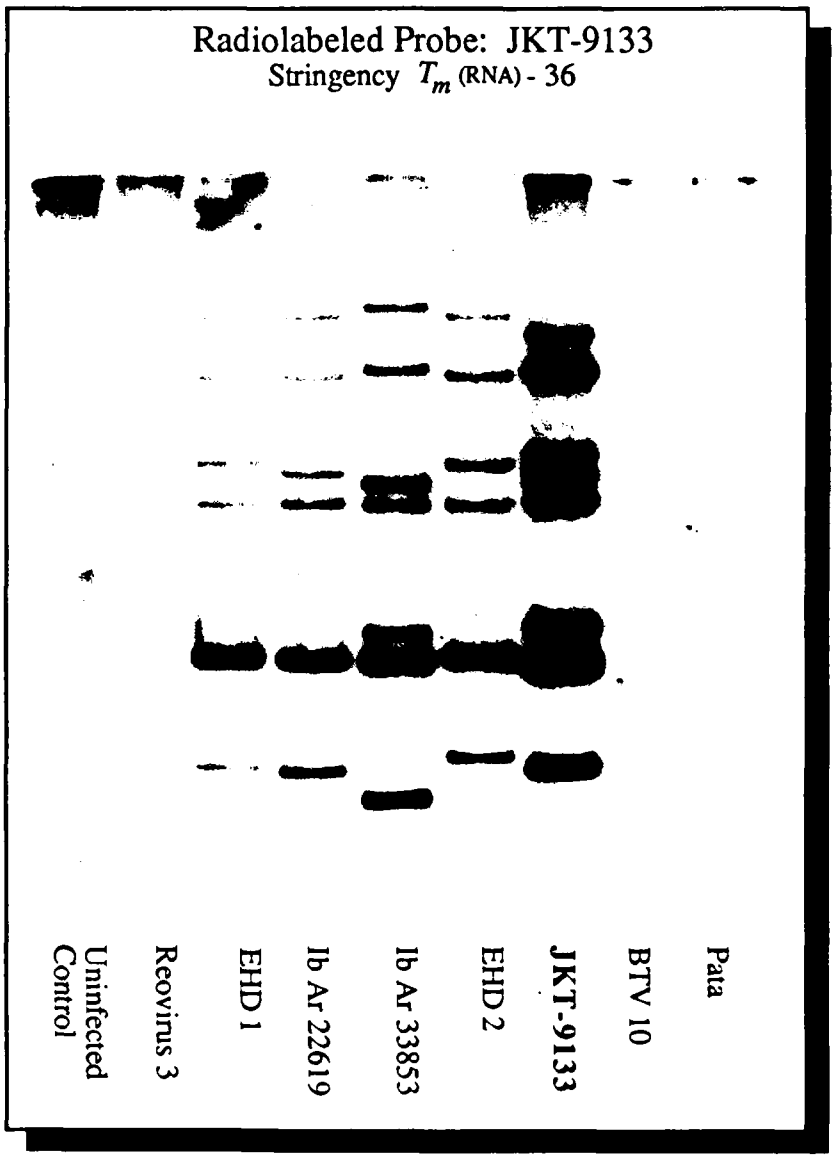
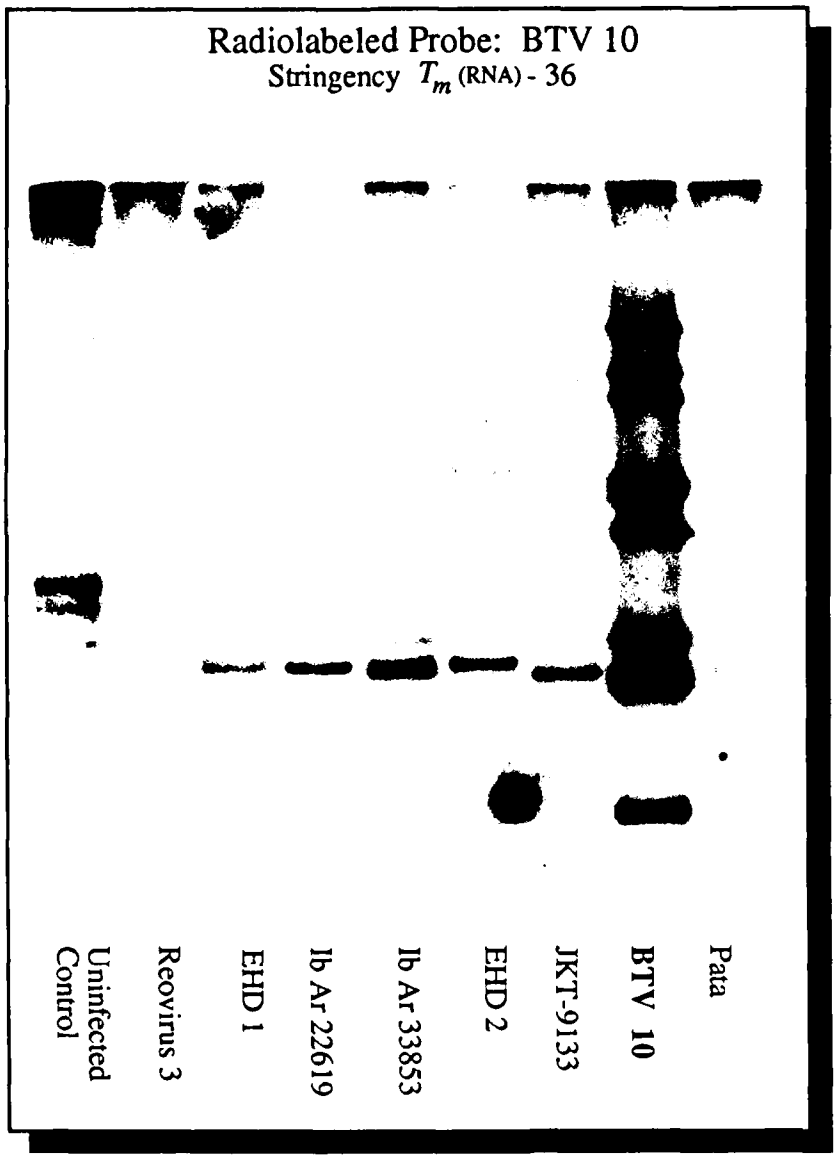


Figure 13. Autoradiograms depicting the hybridization of the following 3' end-labelled genomic dsRNA probes EHD 1 (Fig. 13a), JKT-9133 (Fig. 13b), BTV 10 (Fig. 13c) to a Zeta-Probe membrane containing the profiles of EHD serogroup viruses, BTV 10, and Pata. The lanes are from left to right uninfected control (Lane 1), reovirus 3 Dearing strain (Lane 2), EHD 1 (Lane 3), IbAr 22619 (Lane 4), IbAr 33853 (Lane 5), EHD 2 (Lane 6), JKT-9133 (Lane 7), BTV 10 (Lane 8) and Pata (Lane 9). After each hybridization experiment, the probe was removed from the blot as described.

Blot Hybridization of EHD, BTV, and Pata



Blot Hybridization of EHD, BTV, and Pata



13c

Evolutionary model for orbiviruses (D.L. Knudson). Our recent quantitative RNA hybridization work redefines the degree of genetic relatedness by gene within and between serological groupings of orbiviruses. Using this approach, we have identified new members of several serogroups (De Oliva and Knudson, 1982; Travassos da Rosa et al, 1984; Knudson et al, 1984; Chastel et al, 1984), defined new serogroups (Tesh et al, 1986), and generated data which are suggestive of the underlying mechanisms for genetic diversity within the genus (Bodkin and Knudson, 1985b; 1986).

The large number of recognized Orbivirus serogroups (Table 7) and the data generated from our recent studies favor a model which describes three distinct biological types of orbiviruses found in nature.

Type 1 - Viruses which are maintained in arthropod-vertebrate cycle and which depend upon the vertebrate for their maintenance. These viruses are subjected to the immunologic pressure of their vertebrate host; their surface antigens change, selecting new viral populations which escape the vertebrate herd immunity. Thus, there is a proliferation of distinct serotypes. The CGL, Palyam, and possibly bluetongue viruses are examples of this biologic type (biotype).

Type 2 - Viruses which replicate in the arthropod and infect vertebrates but which are sheltered from the strong selective pressure of the vertebrate immune system. For these viruses, the vertebrate is a replicative dead-end. The vertebrate host may be used in local amplifications of the virus, but the host does not exert any strong selective pressure on the viral gene pool. Since a long-lived arthropod and/or transovarial transmission may also be required for the maintenance of the virus in nature, there is little pressure on the viral gene pool to change its surface antigens. Hence, these viruses may exhibit only a few serotypes, or less distinctive serotypes. Colorado tick fever, Corriparta, and Kemerovo viruses are examples of this biotype.

Type 3 - Viruses which are maintained almost exclusively in arthropods. These viruses may or may not be pathogenic for vertebrates. Yet, they represent a potential Orbivirus gene pool which frequently goes undetected because the traditional viral isolation procedures select for viruses which are pathogenic for vertebrates. Netivot virus (Tesh et al, 1986) and the mosquito isolates are examples of this biologic type.

In nature, there are several mechanisms which influence the degree of genetic diversity in the genus, Orbivirus; the induction of these mechanisms for the speciation of orbiviruses correlates with the biology of the arthropod which harbors and/or vectors the virus. The mechanisms which influence orbivirus speciation include: 1) Genetic drift or point mutations, 2) genetic shift or the physical reassortment of dsRNA segments, and 3) intragenic sequence rearrangement. The contribution which each mechanism makes to the genetic variability of orbiviruses is also correlated with specific viral genes, that is, different viral genes may have different mechanisms which drive their evolution. Each Orbivirus serogroup may represent a gene pool which evolves as a unit or species with mechanism 1 playing the most significant role in the generation of intra-serogroup genetic diversity. However, certain viral genes evolve faster than others

and mechanisms 2 and 3 are responsible for their genesis. The degree with which mechanisms 2 and 3 influence specific gene evolution may well correlate with the biology of the system.

As Della-Porta (1985) has suggested, the time for the considering larger groupings or a higher level of taxonomic distinction is at hand. Let us hope that the scheme reconciles our existing data and that it reflects a better understanding of the biological systems.

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V. EXPERIMENTAL PATHOGENESIS

Studies on the viremia and immune response with sequential phlebovirus infections (R.B.Tesh and S.M.Duboise) Four groups of hamsters were infected sequentially with various combinations of Arumowot, Chagres and Gabek Forest viruses. Following each infection, the survival, level of viremia and immune response of the animals were monitored. Incomplete results were presented in last year's report; the completed results are shown here.

Experiment 1

In the first experiment (Table 28), six normal hamsters were given Gabek Forest virus as their primary infection. On the second and third days post-inoculation, all of the animals had high levels of virus in their blood ($10^{7.6}$ - $10^{9.6}$ PFU/ml). By the fourth day, all of the hamsters were dead. In these non-immune animals, Gabek Forest virus produced a fulminating and uniformly fatal disease.

Experiment 2

The six hamsters in this experiment were infected sequentially at 3-4 week intervals with Arumowot, Chagres and Gabek Forest viruses. The levels of viremia and neutralizing antibody titers developing in the animals after each infection are shown in Table 29. Primary Arumowot virus infection produced a viremia of 3 to 4 days duration, with maximum virus titers ($10^{5.0}$ - $10^{7.0}$ PFU/ml) occurring on the second and third days post-inoculation. All of the animals survived, and the neutralizing antibody response was quite specific.

Three to four weeks later, when the hamsters were given their second infection with Chagres virus, they again developed viremia. Although the level and duration of Chagres viremia was less than that observed during Arumowot virus infection, the level of virus present in the blood after the second infection was not significantly different from the viremia developed by hamsters whose primary infection was with Chagres virus (Experiment 3 - Table 30). These data suggest that previous infection with Arumowot virus did not modify the viremia developing in the animals after Chagres infection. The titer of Chagres virus neutralizing antibodies present in the hamsters' convalescent sera after the second infection also indicates that their immune response was not altered. It is interesting that three of the hamsters in this experiment (animal #2229, 2235 and 2261) showed a rise in neutralizing antibody titers to Arumowot virus after the second infection, suggesting that Chagres virus had a booster effect. It is also noteworthy, that even after the second phlebovirus infection, none of the hamsters had detectable levels of neutralizing antibodies to Gabek Forest virus.

Of the six hamsters which were inoculated with Gabek Forest virus in the third phase of this experiment, only two died. Hamster #2235 died on the fourth day after infection. Unfortunately no blood sample was obtained prior to death, but it seems probable that this animal developed a fulminating infection. Hamster #2234 was found dead two weeks after inoculation with Gabek Forest virus, and it most likely died of other causes. The fact that no virus was

detectable in the animal's blood on the fourth day of infection supports this view. Of the remaining four animals (#2229, #2231, #2261 and #2262), virus was only detected in hamster #2261. The level of viremia in this animal was considerably less than the levels observed in Gabek Forest-infected hamsters in Experiment 1 (Table 28). No virus was detected in the blood of the other three animals (#2229, 2231 and 2262); however, they were only sampled on one or two days after infection and a transient viremia could easily have been missed. Nonetheless, all of the surviving animals in this experiment had neutralizing antibodies against Gabek Forest in their convalescent sera, indicating that they had been infected. The latter data imply that immunity to Arumowot and Chagres viruses somehow modified the third (Gabek Forest) infection.

Experiment 3

The eight hamsters in this experiment (Table 30) were infected sequentially with Chagres, Arumowot and Gabek Forest viruses, respectively. The viremias developed by the animals during their primary Chagres infection were similar to those observed in hamsters in Experiment 2 (Table 29, Second infection). Three weeks after their primary infection, the animals only had neutralizing antibodies to Chagres virus.

During their second phlebovirus (Arumowot) infection, the viremias developed by these Chagres -immune animals were similar to those observed in hamsters initially infected with Arumowot virus (Tables 29 and 30). These data again indicate that the previous Chagres virus infection did not modify the second infection with Arumowot virus. Similarly, all of the hamsters had neutralizing antibodies to Arumowot virus in their convalescent sera (Table), demonstrating a normal immune response to the second infection (Hamster #2236 died before a second convalescent serum could be obtained). It is noteworthy that none of the surviving hamsters had detectable levels of Gabek Forest neutralizing antibodies in their second convalescent sera.

Upon challenge with Gabek Forest virus (third infection), the remaining seven hamsters in this group all developed viremia. Hamster #2237 developed a fulminating Gabek Forest virus infection and died on the fourth day; hamsters #2263 and #2264 had similar fatal infections and died on day 5. The other four animals (#2238, #2241, #2265 and #2270) also developed viremias, but they survived infection and subsequently developed Gabek Forest virus neutralizing antibodies in their convalescent sera. Although the hamsters in this experiment were only bled once or twice, the data (Table 30) suggest that viremia levels in challenged animals were lower than in hamsters with primary infection.

Experiment 4

The hamsters in this experiment (Table 31) were initially infected with Arumowot virus. Three weeks later, they were challenged with Gabek Forest virus. The purpose of the experiment was to determine whether a single phlebovirus infection would protect the animals from the lethal effects of Gabek Forest virus.

The viremia and immune response of the eight hamsters to initial Arumowot virus infection were similar to those observed with the same agent in Experiments 2 and 3 (Tables 29 and 30). When challenged with Gabek Forest virus three to four weeks later, all of the hamsters developed viremia, and six of the

eight (75%) animals died. From the data given in Table 31, it can be seen that levels of viremia in the six dying hamsters were higher than levels detected in the two survivors (#2256 and #2259). It is also interesting that hamsters #2239 and #2257 died on the sixth and seventh days after infection, respectively, suggesting that their immunity to Arumowot provided partial protection and somehow delayed the lethal effects of Gabek Forest virus infection. It is also noteworthy that although the two surviving animals in this experiment (#2256 and #2259) developed Gabek Forest neutralizing antibodies after infection their second convalescent sera had no detectable neutralizing activity against Chagres virus.

The purpose of this study was twofold: (1) to determine the specificity of the immune response following single and multiple phlebovirus infections and (2) to investigate whether cross-protection or immune enhancement might occur with sequential phlebovirus infections. Regarding the first question, the PRNT results from Experiments 2, 3 and 4 (Tables 29-31) indicate that the neutralizing antibody response to phlebovirus infection is quite specific, even after two sequential infections. These data are in contrast to results obtained with other commonly used serologic techniques (hemagglutination-inhibition, complement-fixation, immunofluorescence and enzyme-linked immunosorbent assay), which show considerable cross-reactivity among the various phlebovirus serotypes. Because of its high specificity, the PRNT appears to be the test of choice for use in serologic surveys and in diagnostic work where precise identification of the infecting agent is desired.

Table 28 (Experiment 1)

Viremia in hamsters following infection with Gabek Forest virus*

	Virus titer in blood post-inoculation**			
Animal number	day 1	day 2	day 3	day 4
2206	0	7.8	9.0	(D)
2207	0	7.7	9.0	(D)
2252	0	8.3	(D)	-
2253	0	8.8	9.2	(D)
2254	0	8.0	9.6	(D)
2255	0	7.6	(D)	-

*Hamsters received $10^{4.6}$ PFU of Gabek Forest virus subcutaneously.
**Virus titer expressed as $\log_{10}$ of PFU/ml. 0=<$10^{1.7}$ PFU/ml.
(D) Animal died.

Table 29 (Experiment 2)

Viremia and neutralizing antibody response in hamsters infected sequentially with Arumowot, Chagres and Gabek Forest viruses*

Animal number	Virus titer in blood post-inoculation** day 1	day 2	day 3	day 4	day 5	Neutralizing antibody titer 3 weeks post-inoculation *** AMT	CHG	GF
A. First infection (Arumowot)								
2229	3.0	6.0	6.7	3.2	0	320	10	10
2231	3.9	5.7	7.0	2.8	0	640	10	10
2234	3.4	6.0	6.2	3.6	0	2560	10	10
2235	5.2	6.2	6.5	1.7	0	160	10	10
2261	2.0	5.5	6.2	2.6	0	160	10	10
2262	0	5.0	6.3	2.7	0	80	10	10
B. Second infection (Chagres)								
2229	0	4.3	5.6	2.0	0	1280	320	10
2231	0	4.2	4.0	0	0	1280	320	10
2234	0	0	0	4.8	3.0	2560	160	10
2235	0	0	4.7	3.6	0	1280	320	10
2261	0	0	5.9	5.3	0	2560	1280	10
2262	0	0	6.0	5.4	0	160	80	10
C. Third infection (Gabek Forest)								
2229	-	-	-	0	-	320	160	40
2231	-	-	-	0	-	2560	320	160
2234	-	-	-	0	-	-	-	-
2235	-	-	-	(D)	-	-	-	-
2261	-	3.0	4.6	-	-	2560	1280	160
2262	-	0	0	-	-	320	1280	40

*Hamsters received $10^{5.4}$PFU of Arumowot, $10^{3.0}$ PFU of Chagres, and $10^{4.6}$ PFU of Gabek Forest viruses subcutaneously.
**Virus titer in blood expressed as $\log_{10}$ of PFU/ml. 0=<$10^{1.7}$ PFU/ml.
***Reciprocal of highest serum dilution producing 90% plaque reduction.
- No serum sample taken or tested.
(D)Hamster died.

Table 30 (Experiment 3)

Viremia and neutralizing antibody response in hamsters infected sequentially with Chagres, Arumowot and Gabek Forest viruses*

Animal number	Virus titer in blood post-inoculation** day 1	day 2	day 3	day 4	day 5	Neutralizing antibody titer 3 weeks post-inoculation *** AMT	CHG	GF
A. First infection (Chagres)								
2236	0	5.6	5.9	0	0	10	2560	10
2237	0	2.2	6.0	5.2	0	10	160	10
2238	0	0	5.2	5.4	0	10	640	10
2241	0	0	0	2.5	5.6	10	2560	10
2263	0	3.6	5.5	5.0	0	10	320	10
2264	0	0	5.0	0	0	10	320	10
2265	0	0	4.5	5.3	0	10	320	10
2270	0	4.4	5.8	0	0	10	640	10
B. Second infection (Arumowot)								
2236	2.3	5.0	6.2	3.5	2.5	-	-	-
2237	2.0	5.3	6.0	3.3	2.2	40	320	10
2238	0	4.9	5.4	2.6	2.2	40	320	10
2241	0	4.4	6.0	0	0	640	2560	10
2263	0	3.2	4.0	5.6	0	40	320	10
2264	2.2	4.0	4.7	4.2	0	40	320	10
2265	2.2	4.8	6.6	5.0	0	80	640	10
2270	0	4.7	6.6	4.8	0	160	160	10
C. Third infection (Gabek Forest)								
2237	-	-	-	7.0(D)	-	-	-	-
2238	-	-	-	5.4	-	320	1280	80
2241	-	-	-	4.8	-	640	320	640
2263	-	5.6	7.8	-	(D)	-	-	-
2264	-	7.0	8.8	-	(D)	-	-	-
2265	-	5.0	6.0	-	-	320	640	80
2270	-	5.2	6.8	-	-	1280	640	320

*Hamsters received $10^{3.0}$ PFU of Chagres, $10^{5.4}$ PFU of Arumowot, and $10^{4.6}$ PFU of Gabek Forest viruses subcutaneously.
**Virus titer in blood expressed as $\log_{10}$ of PFU/ml. 0 = $10^{1.7}$ PFU/ml.
***Reciprocal of highest serum dilution producing 90% plaque inhibition.
- No serum sample taken or tested.
(D)Hamster died.

Table 31(Experiment 4)

Viremia and neutralizing antibody response in hamsters infected sequentially with Arumowot and Gabek Forest viruses*

Animal number	Virus titer in blood post-inoculation** day 1	day 2	day 3	day 4	day 5	Neutralizing antibody titer 3 weeks post-inoculation *** AMT	CHG	GF
A. First infection (Arumowot)								
2239	3.3	5.7	6.0	3.4	2.2	640	10	10
2219	3.6	4.7	3.9	2.3	0	80	10	10
2256	3.2	4.7	6.3	3.0	0	160	10	10
2257	2.6	6.0	6.8	3.4	0	160	10	10
2259	2.0	4.4	6.3	0	0	160	10	10
2266	0	5.6	6.9	5.6	2.7	160	10	10
2268	0	6.6	6.3	5.3	0	40	10	10
2269	0	5.7	6.9	5.7	0	80	10	10
B. Second infection (Gabek Forest)								
2239	-	-	-	7.0	- (D)+	-	-	-
2219	-	9.5 (D)	-	-	-	-	-	-
2256	-	5.3	5.8	-	-	320	10	160
2257	-	3.8	6.3	-	- (D)+	-	-	-
2259	-	5.2	5.7	-	-	160	10	40
2266	-	8.5	9.5 (D)	-	-	-	-	-
2268	-	7.2	9.0	(D)	-	-	-	-
2269	-	9.0	(D)	-	-	-	-	-

*Hamsters received $10^{5.4}$ PFU of Arumowot and $10^{4.6}$ PFU of Gabek Forest viruses subcutaneously.
**Virus titer in blood expressed as $\log_{10}$ of PFU/ml. 0 = $<10^{1.7}$ PFU/ml.
***Reciprocal of highest serum dilution producing 90% plaque inhibition.
- No serum sample taken or tested.
(D)Hamster died.
+ Hamster #2239 and 2257 died 6 and 7 days respectively, after infection with Gabek Forest virus.

VI. DISTRIBUTION OF REAGENTS, WHO COLLABORATING CENTRE FOR REFERENCE AND RESEARCH (D.L. Knudson, A.J. Main, R.E. Shope, R.B. Tesh, and G.H. Tignor)

Distribution of reagents: A total of 238 ampoules of virus stocks (114), antigens (59), and antisera (65) were distributed to laboratories in 14 countries plus 8 states in the USA. These included reagents to 92 different serotypes of arboviruses.

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